INTRODUCTION

Dengue is the most important arthropod-borne disease in humans. An estimated 100 million cases of dengue hemorrhagic fever (DHF) and over 500,000 deaths occur each year, including about 2,500 fatal cases that occur mostly in children (1). The main mosquito vector, Aedes aegypti, is present in nearly every tropical country, and consequently, a third of the world’s human population is at risk of infection. Dengue virus infection is mostly asymptomatic but can produce a mild, self-limiting acute febrile illness, dengue fever, or a life threatening severe illness, dengue hemorrhagic fever. Dengue hemorrhagic fever is associated with increased vascular permeability partly as a result of elevated levels of matrix metalloproteinases (MMPs). We characterized MMP-2 and MMP-9 production in mosquito and mammalian cells after infection with three strains of dengue virus type-2 (D2-) ranging in virulence: 16681, the prototype New Guinea C (NGC), and PDK-53 vaccine strain. These strains were used to test variations in viral properties in vaccine candidates and confirm the production of MMP as a possible marker for virulence. A zymogram gelatinolytic activity assay was used to assess MMP-2 and MMP-9 production patterns depending on the virulence of the infecting dengue strain and the duration of infection. MMP levels were highest after infection with the most virulent strain D2-16681, followed by the prototype NGC strain, in both cell lines. The MMP levels appeared to correspond with the relative amounts of infectious virions produced later in infection. Our findings improve our understanding of dengue pathogenesis and may facilitate the selection of markers to further the development of dengue vaccines.

SUMMARY: Dengue virus infections are mostly asymptomatic but can produce a mild, self-limiting acute febrile illness, dengue fever, or a life threatening severe illness, dengue hemorrhagic fever. Dengue hemorrhagic fever is associated with increased vascular permeability partly as a result of elevated levels of matrix metalloproteinases (MMPs). We characterized MMP-2 and MMP-9 production in mosquito and mammalian cells after infection with three strains of dengue virus type-2 (D2-) ranging in virulence: 16681, the prototype New Guinea C (NGC), and PDK-53 vaccine strain. These strains were used to test variations in viral properties in vaccine candidates and confirm the production of MMP as a possible marker for virulence. A zymogram gelatinolytic activity assay was used to assess MMP-2 and MMP-9 production patterns depending on the virulence of the infecting dengue strain and the duration of infection. MMP levels were highest after infection with the most virulent strain D2-16681, followed by the prototype NGC strain, in both cell lines. The MMP levels appeared to correspond with the relative amounts of infectious virions produced later in infection. Our findings improve our understanding of dengue pathogenesis and may facilitate the selection of markers to further the development of dengue vaccines.

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virus-infected immature dendritic cells (DCs) overproduced soluble MMP-9, resulting in enhanced endothelial permeability (5). We also showed that infection of primary human microvascular endothelial cells resulted in the overproduction of MMP-2 and to a lesser extent MMP-9, leading to enhanced endothelial permeability (15). Both of these findings which support the molecular basis and involvement of MMPs in dengue pathogenesis. The aim of this study was to characterize MMP-2 and MMP-9 production in mosquito and mammalian cells after infection with 3 strains of dengue virus type-2: 16681, the prototype New Guinea C (NGC), and PDK-53 (vaccine strain). We used these three strains of variable virulence to test variations of viral properties in vaccine candidates and to confirm that the production of MMP as a possible virulence maker.

**MATERIALS AND METHODS**

**Cell lines and dengue viruses:** The larval stage cell line of *Ae. albopictus*, C6/36, was maintained at 28°C and 5% CO₂ in Minimum Essential Medium with 5% FBS. The rhesus monkey kidney cell line, LLC/MK2, was grown at 37°C and 5% CO₂ in M199 media and 5% FBS. The 3 strains of dengue type-2 used in the study included 2 wild type strains, 16681 (as virulence strain) and NGC (as non-virulence strain) (16). A vaccine derivative strain from D2-16681, PDK-53 (17) was also included. Each strain was propagated in the C6/36 mosquito cell line and supernatants were collected when cells reached approximately 30% confluency (approximately 7 days after incubation). Supernatants were stored at −80°C prior to performing the plaque titration assay.

**Replication kinetics of dengue virus infection in C6/36 and LLC/MK2 cells:** C6/36 and LLC/MK2 cells (1.6 × 10⁵) were infected with each dengue virus type-2 at a multiplicity of infection (MOI) of 1 in a final volume of 3 ml. Cells were seeded in the appropriate complete media 3 days prior to infection. On the day of infection, one flask of control cells was counted to calculate the appropriate MOI. Briefly, viruses were incubated for 2 h at 28°C or 37°C and 5% CO₂. Cells were washed twice and 15 ml of complete medium was then added to each flask. Cells were returned to the incubator for the duration of the infection experiment. During the incubation period, 300 μl of cell supernatant was collected at each time point (0 to 15 days after infection) and stored at −80°C prior to performing the plaque titration assay.

**Plaque titration assay:** LLC/MK2 cells were seeded in 12-well plates 2–3 days prior to infection and maintained in complete medium M199. On the day of the assay, 10-fold serial dilutions of supernatants collected from infected C6/36 or LLC/MK2 cells were prepared (100 μl of virus in 900 μl of medium). After washing LLC/MK2 cells with PBS, 100 μl of the appropriate viral dilution was added, and the cell were then incubated for 90 min at room temperature. Cells were overlaid with 1.8% low melting point agar and an overlay medium at equal volumes. Overlaid cells were then incubated at 37°C and 5% CO₂ for 7 days. After incubation, cells were overlaid with equal volumes of 1.8% low melting point agar and a second overlay medium mixture containing 4% neutral red. The plates were incubated overnight at 37°C and 5% CO₂. The results of plaque titration were read within 24 h. Plaque titrations of all strains of the D2-viruses were performed in triplicate. The sizes of 100 plaques were measured, and the average size of the triplicates was used to compare the groups using Student’s t-test.

**Reverse transcriptase polymerase chain reaction (RT-PCR) and nested-PCR:** Dengue virus RNA was extracted from C6/36 cell supernatants using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s protocol. Isolated RNA was eluted into 50 μl of buffer. The RNA template was reverse-transcribed into cDNA and then amplified using the One-Step RT-PCR kit (Qiagen) following a manufacturer’s protocol. Briefly, 5 μl of extracted RNA was added to 20 μl of RT-PCR mixture. Amplification occurred in a PCR thermal cycler at 42°C for 60 min followed by 35 cycles of denaturation (94°C, 30 s), primer annealing (55°C, 1 min), and extension (72°C, 2 min). Five primer sets were used for the nested-PCR: D1 and 4 internal dengue type specific primers (TS1bis, TS2, TS3, and TS4) as previously published (18). Briefly, 5 μl of 1:50 diluted cDNA from the initial RT-PCR was mixed with 20 μl of the nested-PCR mixture and amplified for 30 cycles of denaturation (94°C, 30 s), annealing (55°C, 1 min), and extension (72°C, 2 min). The PCR products were detected by agarose gel electrophoresis with ethidium bromide staining. The gel was digitized using Epson scanner expression 10000 XL (Epson, Long Beach, CA, USA).

**Detection of MMP activity by gelatinolytic activity (zymogram) and MMP quantification:** MMP-2 and MMP-9 were detected in the supernatants of C6/36 cell and LLC/MK2 cells using gelatin zymography as previously described (19). Briefly, 1.6 × 10⁵ C6/36 cells and 3.4 × 10⁵ LLC/MK2 cells were cultured in 24 well plates and infected with each of the 3 dengue virus type-2 strains at an MOI of 1 for 1 h. The supernatant was collected at 0.5, 1, 6, 12 and 24 h after infection and was concentrated by lyophilization. Approximately 80 μg of protein was loaded into the wells of a polyacrylamide gel containing 7.5% (weight per volume, w/v) acrylamide incorporated with gelatin (1 mg/ml) and a stacking gel containing 4% (w/v) acrylamide (Bio-Rad, Hercules, CA, USA). Electrophoresis was performed at 120 volts under non-reducing conditions. The gel was prepared for visualization by staining with Coomassie Brilliant Blue 250-R followed by de-staining. Zones of enzyme activity were detected as unstained regions. MMP-2 was detected as a 72 kDa band (gelatinase A) and MMP-9 was detected as a 92 kDa band (gelatinase B). Gelatinolytic activity was quantified by densitometry using the Gene Genius Gel documentation system with Genetools software version 4.0 (Syngene, Cambridge, UK). Relative MMP-2 and MMP-9 expression levels were calculated using the sample with the highest expression level as the control (the 1 h time point for C6/36 infected cells and the 0.5 h time point for LLC/MK2 cells). Statistical analyses, including the Student t-test, were performed to investigate the significance of the observed differences in MMP expression levels across viral strains.
RESULTS

Dengue virus replication kinetics in mosquito and mammalian cell lines: The growth characteristics of D2-16681, D2-NGC, and D2-PDK-53 strains were compared in mammalian LLC-MK2 cells and mosquito C6/36 cells. The D2-16681 strain demonstrated the highest replication rate in both cell lines among the 3 strains followed by D2-NGC and D2-PDK-53, respectively (Fig. 1A and B). Peak D2-16681 replication occurred 3 days after infection in LLC/MK2 cells when viral production reached over 8.5 log PFU/ml and peaked at 5 days after infection in C6/36 cells when viral production reached 8.0 log PFU/ml. The plaque phenotype as a result of dengue virus type-2 (DENV-2) infection was determined by measuring the mean diameters of the plaques produced by infectious virions isolated from the supernatants of infected mammalian and mosquito cells (Fig. 2). D2-16681-infected LLC-MK2 cells demonstrated the largest plaques sizes. These plaques averaged 2.2 mm and ranged from 1.3 to 3.1 mm in diameter. Plaques generated from the D2-PDK-53 in LLC-MK2 cells were smaller: these averaged 1.8 mm and ranged from 1.1 to 2.6 mm in diameter. Plaques formed in infected C6/36 cells were smaller than LLC/MK2-infected cells overall. Plaque sizes were not significantly different.

Viral RNA was purified from the supernatant and amplified by RT-PCR. The dengue virus was confirmed by agarose gel electrophoresis of a 511 base pair product generated by flavivirus-specific consensus primers (data not shown).

MMP-2 and MMP-9 production characteristics in DENV-2 infected mosquito and mammalian cells: The supernatants from the DENV-2 infected C6/36 and LLC/MK2 cells were collected at 0.5, 1, 6, 12, and 24 h after infection to detect MMP-2 and MMP-9 production via a zymogram gelatinolytic activity assay (Fig. 3A and 4A). The quantities of MMP detected in the zymogram bands were determined by densitometry. The levels of MMP in C6/36 cells are reported as percent relative to the band of MMP produced by D2-16681 at 1 h after infection for both MMP-2 and MMP-9. The levels of MMP in LLC/MK2 cells are percent relative to the band of MMP produced by D2-16681 isolated 0.5 h after infection. These values are reported in Fig. 3B and 4B, and those are illustrated as graphs (Fig. 3C and 4C). The D2-16681 and D2-NGC strains induced MMP-2 and MMP-9 in C6/36 cells (Fig. 3A). More MMP-2 was detected in the supernatant of D2-16681-infected C6/36 cells than D2-NGC-infected C6/36 cells at each time interval. MMP-2 production began at the first hour after infection, then decreased continuously. MMP-2 was absent at 24 h after D2-16681 infection and 12 h after D2-NGC infection. D2-NGC-infected C6/36 cells produced MMP-2 at only 2 time points, 1 and 6 h after infection (Fig. 3A). MMP-2 production in D2-NGC-infected C6/36 cells was 69% of that in D2-16681-infected cells at 1 h after infection. This gap in MMP-2 production widened at 6 and 12 h after infection, diminishing to 15% and 0% in D2-NGC-infected cells, however, in D2-16681-infected cells, to 67% and 53%, respectively (Fig. 3B and C).

MMP-9 production in C6/36 cells was high at all-time points after D2-16681 infection. Production peaked early at 0.5 h after infection, decreased to 12 h, and slightly increased at 24 h after infection (Fig. 3B and C). Simi-
Fig. 3. MMP in the supernatants of dengue virus type-2 infected C6/36 cells. (A) Zymograms of MMP-2 and MMP-9 present in the supernatants of C6/36 cells infected with D2-16681, NGC, or PDK-53 strains at designated time points after infection. MMP-2 and MMP-9 are identified as the 72 and 92 kDa de-stained bands, respectively. (B and C) Genetools software was employed to detect the optical density of MMP-2 and MMP-9 activities from the zymogram. Gelatinase activities and the ratio to that of D2-16681 at 1 h after infection are represented in the table. *, significantly different to both of D2-NGC and D2-PDK-53 (P < 0.05); **, significantly different to only D2-PDK-53 (P < 0.05).

MMP-9 production followed the trend of MMP-2 production in LLC/MK2 cells for all 3 dengue strains (Fig. 4B and C). D2-PDK-53-infected cells produced lower levels of MMP-9 than both D2-16681- and D2-NGC-infected cells all-time points. MMP-9 was detectable at 6, 12, and 24 h after infection, unlike MMP-2. Overall, these results demonstrate that different dengue virus type-2 strains are able to induce different amounts of MMPs depending on the virulence of the virus.

DISCUSSION

In this study, we demonstrated that dengue virus type-2-infected mosquito and mammalian cell lines had unique MMP-2 and MMP-9 production patterns that depended on the virulence of the virus and on the time since infection. The amounts of MMP-2 and MMP-9 appeared to correspond with the relative amounts of infectious virion production. MMP levels were highest after infection with the most virulent strain, D2-16681, followed by the prototype NGC strain in both cell lines. Our in vitro results support previous clinical findings that MMP-9 is associated with disease severity in dengue fever (3) and that increased MMP-2 levels are significantly associated with plasma leakage in DHF patients (20). In vitro studies have demonstrated that MMP overproduction as a result of dengue virus infections in endothelial cells or DCs can lead to increased cell permeability (5,15). Together, these findings suggest...
that the theory of dengue pathogenesis, in which virulent dengue strains more efficiently infect cells and produce vasoactive mediators including MMPs is indeed relevant to severe dengue disease progression.

The dengue virus phenotype from both cell lines measured by plaque size, and the viral replication rates were determined. D2-16681 infections produced the largest plaques of the three strains. These differences in plaque size suggest a tendency of the non-attenuated D2-16681 strain to cause more cell damage than the prototype and vaccine strains. A previous study demonstrated that mutated dengue virus type-2 strains with attenuated serine proteinase activity produced smaller plaques and fewer virions (21). All three strains of dengue virus replicated in both kinds of cells; however, the replication rates differed depending on the virus strain used and the cell type infected. Where a more viral rapid replication rate was observed in the mammalian cells after infection with D2-16681, the C6/36 cell line supported a more sustained infection from days 3–7 after infection. The sustained infection may be due to the fact that C6/36 cells harbor a specific laminin-binding protein receptor that may enhance the entry of dengue viruses into the cell (22,23). Furthermore, MMP-2 and MMP-9 were able to enhance viral entry into cells and have been correlated with the pathogenesis of infection (24,25). As such, replication rates observed in both cell types were sustained as a result of the early induction of MMP-9 in C6/36 cells and MMP-2 in mammalian cells. Others previously observed that dengue infected primary human endothelial cells (mammalian cells) resulted in the overproduction of MMP-2 and to a lesser extent of MMP-9, leading to enhanced endothelial permeability in vitro. This permeability was associated with loss of expression of the vascular endothelium-cadherin cell-cell adhesion and may contribute to the pathogenesis of severe dengue infection (15). This finding supports our results that demonstrate higher absolute and sustained levels of MMP-2 in mammalian cells upon dengue infection.

MMP-2 and MMP-9 levels were higher in both types of cells after infection with D2-16681 compared to levels in cells infected with D2-NGC or D2-PDK-53, suggesting that MMP levels may correspond to viral replication kinetics and may be based on the amount of viral antigens during replication at very early stages. Our results demonstrate that MMP levels increased at 24 h after infection, suggesting that translation of viral proteins may sustain MMP production. In fact, MMP-9 activity has been shown to be dependent on viral load (5) and increased MMP gene transcription can be modulated by a large variety of soluble factors, including cytokines, growth factors, and hormones, or by cellular contacts acting through specific signaling pathways (26). It is also possible that increased MMP levels 24 h after infection are due to the recovery of cells or reinfection of progeny virus to viable cells. Infection by the D2-16681 strain resulted in increased levels of MMPs apparently as a consequence of cell apoptosis. Overall, the more virulent strains enhanced entry of the virus into cell,
possibly as a result of structural characteristics (27). MMP-2 production from all 3 strains tended to be higher than MMP-9, possibly because of the nature of LLC/MK2 cells that predominantly secrete MMP-2. Further studies are needed to better understand the link between the apopotosis of C6/36 cells and MMP production.

Cytokines predominately found in the plasma of DHF patients, interleukin-8 and tumor necrosis factor-α (TNF-α), can induce MMP-2 and MMP-9 secretion via the mitogen activated protein-kinase pathway (28,29). TNF-α-induced MMP-9 expression, secretion, and activity were demonstrated by others to be completely blocked by a stress-activated protein kinase/Jun kinase and extracellular signal-regulated kinase inhibitors (29). Although the signaling pathways that lead to the induction of MMP expression during dengue infection are still incompletely understood, certain patterns are beginning to emerge. Insights into the mechanism controlling MMP production might be useful to explore a general MMP inhibitor that could be used during severe dengue infections.

Others have suggested that MMPs may be used as a biological marker of dengue infection severity because MMP levels in recipients of candidate dengue vaccines may be important markers to consider. Interestingly, MMP levels in recipients of candidate dengue vaccines biologically marker of dengue infection severity because of the mitogen activated protein-kinase pathway (28,29).

Blockage of MMP production might be useful to explore a general MMP inhibitor that could be used during severe dengue infections.

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Conflict of interest

None to declare.

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