Cell entry mechanisms of dengue virus
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2009

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
General introduction
Dengue virus (DENV) currently causes the most common mosquito-borne viral infection in the world. According to the estimates of the World Health Organization, 50 to 100 million infections occur each year leading to 500,000 hospitalizations and 20,000 deaths (WHO, 2009). Around 2.5 billion people live in areas infested with the domesticated mosquito *Aedes Aegypti*, which transmits the virus, and thus are at risk for infection, see Figure 1. During the last few decades, the incidence of DENV infections has increased dramatically, which can be attributed to major societal and economical changes, including population growth, urbanization, lack of mosquito control, international trade, and modern transportation. These factors have promoted the geographical spread of all four DENV serotypes throughout the (sub)tropical regions of the world, which has resulted in larger and more severe outbreaks (Gubler, 2002; Mackenzie et al., 2004).

DENV, an enveloped positive-sense RNA virus, is classified to the Flavivirus genus within the Flaviviridae family, which also includes yellow fever virus, West Nile virus, and tick-borne encephalitis virus. There are four closely related serotypes of DENV, called DENV-1 to DENV-4 (Lindenbach & Rice, 2001). Although many DENV infections are clinically inapparent,
each serotype can cause disease in humans. Most individuals that do become ill experience uncomplicated dengue fever, an acute febrile illness accompanied by headache, often muscle and joint pain, nausea, vomiting, and rash. Yet, approximately 500,000 patients per year develop potentially life-threatening dengue hemorrhagic fever (DHF), which is characterized by high fever, plasma leakage, bleeding manifestations, and often hepatomegaly. In severe cases, plasma leakage can result in circulatory failure and hypovolemic shock, which is called dengue shock syndrome (DSS). Without proper treatment, such as effective replacement of the plasma loss, DHF/DSS can be fatal (Gibbons & Vaughn, 2002; Halstead, 2007; Rigau-Perez et al., 1998). Despite the high clinical impact of DENV, there are neither vaccines nor antiviral drugs available yet to prevent or treat the disease.

The pathogenesis of DHF/DSS is not completely understood. Several clinical studies have established factors that correlate with disease severity. First of all, there is a considerably larger dengue-infected cell mass and higher viremia titers in the prelude of DHF (Libraty et al., 2002; Vaughn et al., 2000; Wang et al., 2003). Also, the serum levels of several pro- and anti-inflammatory cytokines and chemical mediators, such as TNF-$\alpha$, IL-$\beta$, IL-2, IL-6, IL-8, IL-10, IL-12, IL-13, and IL-18, are higher in patients with DHF (Bethell et al., 1998; Braga et al., 2001; Green et al., 1999b; Hober et al., 1993; Kurane et al., 1991; Mustafa et al., 2001; Pacsa et al., 2000; Raghupathy et al., 1998). Furthermore, lymphocytes isolated from DHF patients show markers of activation, such as expression of CD69 and secretion of soluble IL-2 receptor, soluble CD4, and soluble CD8 (Green et al., 1999a; Kurane et al., 1991). Other clinical studies have demonstrated that high serum levels of complement factor C3a and the SC5b-9 complement complex are associated with DHF as well (Avirutnan et al., 2006; Malasit, 1987). The elevated vascular permeability causing plasma leakage is presumably the consequence of a functional disturbance of the endothelial cells, most likely induced by the cytokine storm, as examination of the capillaries in skin biopsies of DHF patients has revealed that there is no severe damage to the endothelial cells (Basu & Chaturvedi, 2008; Sahaphong et al., 1980).

What is the cause of the high viral load and the strong immune response in the onset of DHF? Intriguingly, many epidemiological studies have demonstrated that the development of DHF is strongly associated with secondary infections with a heterotypic serotype, i.e. a serotype different than the one(s) the individual has encountered before (Burke et al., 1988; Guzman et al., 1990; Halstead et al., 1969; Sangkawibha et al., 1984; Thein et al., 1997).
Furthermore, infants born to dengue-immune mothers have been shown to be at greater risk for developing DHF during a primary infection due to passively transferred maternal antibodies (Halstead et al., 2002; Kliks et al., 1988; Kliks et al., 1989; Nguyen et al., 2004; Simmons et al., 2007). Together these studies have led to two compatible hypotheses on enhancement of DENV infection that describe the role of antibodies and T cells in the development of severe disease (Fink et al., 2006; Halstead, 2007; Lei et al., 2001; Rothman & Ennis, 1999; Rothman, 2004). The first and widely accepted hypothesis concerns antibody-dependent enhancement (ADE) of DENV infection. ADE occurs when pre-existing antibodies direct the newly infecting virus particles towards the cells of the immune system, which are the natural host cells of DENV (Halstead, 2003; Kou et al., 2008; Pang et al., 2007; Rothman & Ennis, 1999; Sullivan, 2001; Wu et al., 2000). The antibodies facilitate more efficient cellular uptake of the virus via Fc receptor-mediated phagocytosis. Yet, the antibodies somehow fail to neutralize the virus intracellularly and thus increase the number of infected cells, which will result in a high viral load and induce the immunological cascade leading to plasma leakage. T cells are subsequently believed to play a pivotal role in this immunological cascade. The other hypothesis postulates that during a heterotypic secondary infection, there is a preferential activation of memory T cells with a higher avidity for primary serotype and thus a lower avidity for the newly infecting virus, a phenomenon called "original antigenic sin", which results in altered T cell effector functions and increased cytokine production (Imrie et al., 2007; Mongkolsapaya et al., 2006; Zivny et al., 1999). Moreover, these cross-reactive memory T cells are expanded more rapidly in a secondary infection than the naive T cell population (Mongkolsapaya et al., 2003), which could drive the immune response towards a more pathological outcome. Taken together, it appears that T cells primarily operate in the strong immune response in the prelude of DHF, while antibodies directly influence the infectious properties of the virus by altering the cell entry mechanisms.

DENV enters its host cell via receptor-mediated endocytosis (Lindenbach & Rice, 2001), see Figure 2. The first step in this process is binding of the virus particle to a cellular receptor. Subsequently, the virus particle is internalized into the cell and transported to an intracellular vesicle called an endosome (Flint et al., 2004). Endosomes maintain an acidic lumen, which induces conformational changes in the major envelope protein E that promote fusion of the viral membrane with the endosomal membrane (Heinz et al., 2004; Kielian & Rey, 2006; Kuhn et al., 2002; Modis et al., 2004; Perera et al., 2008; Rey et al., 1995). Upon membrane fusion, the nucleocapsid enclosing the viral
genome is delivered into the cytosol to initiate viral replication. Following viral RNA replication and translation, immature virions are assembled by budding of newly formed nucleocapsids into the lumen of the ER, thereby acquiring a lipid bilayer envelope with the structural proteins prM and E (Lindenbach & Rice, 2001), see Figure 2. Subsequently, the immature virions mature during transport through the acidic trans-Golgi network, where the prM proteins are believed to stabilize the E proteins to prevent conformational changes that could lead to premature abortion of the productive cycle (Guirakhoo et al., 1992; Heinz et al., 1994; Li et al., 2008; Lorenz et al., 2002). Shortly before release of the virions from the host cell, the maturation process is completed when prM is cleaved by the cellular protease furin into a soluble pr peptide and virion-associated M. Upon secretion from the cell, the virus particles encounter a neutral pH, which promotes dissociation of the pr peptides from the virus particles and generates mature, infectious virions (Elshuber et al., 2003; Guirakhoo et al., 1991; Perera et al., 2008; Stadler et al., 1997).
Remarkably, several *in vitro* studies have demonstrated that, compared to other flaviviruses, cells infected with DENV release high numbers of immature particles containing unprocessed prM (Anderson et al., 1997; He et al., 1995; Henchal et al., 1985; Murray et al., 1993; Putnak et al., 1996; Randolph et al., 1990; Wang et al., 1999; Zybert et al., 2008). Multiple studies on other immature flavivirus particles have indicated that the maturation process is a prerequisite for viral infectivity (Elshuber et al., 2003; Heinz et al., 1994; Stadler et al., 1997). Indeed, immature DENV particles appear non-infectious in titration assays (Zybert et al., 2008), which might imply that these particles are an irrelevant byproduct of DENV infection. Interestingly, antibodies directed against prM are commonly found in sera of patients (Bray & Lai, 1991; Cardosa et al., 2002; Lai et al., 2008; Se-Thoe et al., 1999). Moreover, the antibody responses to prM are significantly elevated in patients after secondary dengue episodes (Lai et al., 2008), which might suggest a contribution of immature DENV to the pathogenesis of severe disease.

To better understand the molecular mechanisms involved in enhancement of DENV infection by antibodies, it is essential to gain fundamental insights into the cell entry properties of the virus. Dissection of the cell entry pathway of DENV will enrich our knowledge on the critical determinants in DENV infection and may contribute to the rational design of safe antiviral drugs and vaccines. Additionally, in order to elucidate the potential role of immature particles in the disease pathogenesis, it is important to study the infectious properties of immature particles in the presence of anti-prM antibodies.
Scope of this thesis

The studies described in this thesis focus on the mechanisms involved in cell entry that control the infectious properties of DENV.

Chapter 2 provides a general overview of the potential mechanisms involved in cell entry of enveloped viruses. Subsequently, the internalization route of flaviviruses, including DENV, is outlined as well as the intracellular trafficking behavior prior to membrane fusion. Since this chapter is published as a review article, results described in Chapters 3 and 4 are also discussed here. Finally, the current hypotheses on how antibodies can either neutralize or enhance viral infection through alteration of the cell entry process of DENV are addressed.

In Chapter 3, the infectious properties of DENV are investigated by determination of the physical particle to infectious unit ratio using several biochemical and titration assays. In order to explain the observed ratio, a method based on real-time fluorescence microscopy is used to visualize the cell entry process of individual DENV particles in living cells. The cellular binding properties of DENV, its intracellular transport behavior and the time point of membrane fusion are examined to determine the critical steps in cell entry that define the infectivity of DENV.

Chapter 4 presents an elaborate study on the cell entry pathway of DENV to elucidate the internalization route, trafficking through the endocytic network, and the organelle where membrane fusion occurs. Single virus particles are simultaneously tracked with endocytic structures illuminated by fluorescent marker proteins in living cells, which enables us to gather unique information on the mechanisms and kinetics in DENV entry.

In Chapter 5, the effect of an anti-prM antibody on the infectious properties of immature DENV particles is investigated in Fc receptor-expressing cells. To this end, the different stages in the cell entry process of antibody-opsonized immature particles are examined. The binding efficiency of immature particles to Fc receptor-bearing cells is determined as well as the involvement of furin in the entry process.

Chapter 6 gives a summarizing discussion of the results and conclusions presented in this thesis.
Chapter 1

References


protein confers acid resistance to virus particles and alters the expression of epitopes within the R2 domain of E glycoprotein. Virology 191: 921-931.


General introduction

Chapter 1


