The role of antibodies in controlling flavivirus cell entry
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INTRODUCTION

Antibodies (Abs) are critical in the protection against and clearance of flavivirus infections (Pan et al., 2001; Diamond et al., 2003; Shrestha et al., 2008). Indeed, in murine models for flavivirus infection, passive transfer of monoclonal antibodies (MAbs) or immune serum protects mice against lethal challenge (Jacoby et al., 1980; Mathews & Roehrig, 1984; Diamond et al., 2003; Goncalvez et al., 2008). Furthermore, several MAbs have been shown to have therapeutic potential in mice when administered several days after otherwise lethal infection, highlighting their importance in clearing the virus from peripheral organs (Kimura-Kuroda & Yasui, 1988; Roehrig et al., 2001; Oliphant et al., 2005; Goncalvez et al., 2008; Shrestha et al., 2010; Sukupolvi-Petty et al., 2010). The Ab response to flavivirus infection is directed against the structural glycoproteins prM and E, and the nonstructural protein 1 (NS-1) (Throsby et al., 2006; Lai et al., 2008; Dejnirattisai et al., 2010). Interestingly, the ability of Abs directed against the E-glycoprotein to potently inhibit viral entry into target cells was found to be an important correlate of protection in vivo (Oliphant et al., 2005; Throsby et al., 2006; Oliphant et al., 2006; Shrestha et al., 2010). At the same time, enhanced uptake of E Ab-opsonized flaviviruses into cells bearing Fc-γ receptors (FcR) has been proposed to be directly involved in DENV pathogenesis through a mechanism known as Ab-dependent enhancement (ADE) of infection (Halstead & O’Rourke, 1977; Halstead et al., 1977; Halstead, 2003; Dejnirattisai et al., 2010; Balsitis et al., 2010). Consequently, a more detailed insight into the entry mechanisms of flaviviruses is crucial for our understanding of antibody-mediated neutralization and enhancement of flavivirus infection. The studies described in this thesis focus on the various steps in the cellular entry pathways of DENV and WNV as important targets for Ab-mediated neutralization or enhancement of flavivirus infection.

STRUCTURAL TRANSITIONS IN THE FLAVIVIRUS LIFECYCLE

Flavivirus particles are dynamic structures. The viral surface proteins undergo large-scale conformational rearrangements during virus assembly and maturation, and their subsequent entry into target cells. In mature virus particles, 90 E-glycoprotein homodimers are orientated perpendicular to the viral surface in a head-to-tail fashion (Kuhn et al., 2002; Zhang et al., 2004). The E-protein itself consists of three domains designated DI – III (Rey et al., 1995; Modis et al., 2003; Nybakken et al., 2006; Kanai et al., 2006). DI consists of an 8-stranded β-barrel and bridges the other two domains through flexible linkers, providing flexibility to the E-protein. Protruding from DI is a finger-like domain termed DII, with the fusion loop (cd-loop) at its distal end. Its extended β-stranded structure is crucial for oligomeric interactions between E-proteins. DIII is an Ig-like domain and anchors the E-protein into the viral membrane through a stem region. In addition, DIII is postulated to contain the receptor-binding domain (Rey et al., 1995; Chen et al., 1997; Lee & Lobigs, 2000).

Upon binding of the E-glycoprotein to the cell surface, the flavivirus particles are internalized through clathrin-mediated endocytosis (Chu & Ng, 2004; van der Schaar et al., 2008) and fuse from within acidic endosomes, thereby releasing its RNA genome into the cell cytosol (van der Schaar et al., 2007; Krishnan et al., 2007; van der Schaar et al., 2008; Sips, Moesker, Wil-
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schut & Smit, unpublished results). In Chapter 3 we investigated the functional requirements for West Nile virus (WNV) membrane fusion using liposomal target membranes. We found that mildly acidic pH is the sole condition required to trigger fusion with liposomes, which is in accordance with earlier studies on WNV and tick-borne encephalitis virus (TBEV) (Gollins & Porterfield, 1986b; Corver et al., 2000). The architecture of the E-protein has evolved to respond to changes in pH through a sensing mechanism that likely involves one or more highly conserved histidine (His) residues (Kampmann et al., 2006; Fritz et al., 2008). Protonation of these His residues, and possibly other residues with ionizable side-chains (Nelson et al., 2009) serves as a ‘pH switch’ that promotes a series of conformational rearrangements of the E-protein lattice on the virion surface. The meta-stable dimeric conformation of the E-glycoprotein is destabilized upon exposure to low pH, causing the dimers to dissociate (Stiasny et al., 1996; Stiasny et al., 2002; Stiasny et al., 2007). Through a monomer intermediate, the E-Protein lattice on the viral surface then rearranges into 60 homotrimeric complexes (Bressanelli et al., 2004; Modis et al., 2004). During this highly orchestrated molecular event, the fusion loops at the tips of the extended monomers are inserted into the target membrane. Refolding of the monomers into trimers allows for DIII and the stem region within each monomer to ‘zipper up’ alongside the trimer interface to form a hairpin (Bressanelli et al., 2004; Stiasny et al., 2005; Liao et al., 2010), thereby bringing the viral membrane and target membrane into close proximity. At this point, the outer layers of the two lipid membranes will merge, forming a so-called hemifusion intermediate. The cooperative association of increasing numbers of activated homotrimeric complexes is believed to be required for progression into a complete fusion pore (Gibbons et al., 2004). This notion is supported by experimental observations suggesting that the most energy-intensive stages in the fusion process follow rather than precede the formation of a hemifusion intermediate (Zaitseva et al., 2005).

As flavivirus particles progress through the endocytic pathway they encounter increasingly lower pH values. In Chapter 3, we show that WNV membrane fusion is most optimal (in terms of the rate and extent of fusion) at pH 6.3. At higher pH values, lower rates and extents of fusion were observed with a pH threshold for fusion of approximately 6.9. The observation that, in our lipid-mixing assay, a smaller fraction of the virus population undergoes fusion at pH 6.7 compared to pH 6.3, suggests that some particles fuse at pH 6.7 while others do not. This apparent heterogeneity may relate to the maturation state of individual particles (as will be discussed below) and their ability to ‘recruit’ activated trimers to participate in the formation of fusion pores at suboptimal pH values (Zaitseva et al., 2005). It would be of interest to investigate the fusion kinetics of WNV particles at these pH values at a single-particle level (Floyd et al., 2008). One possible outcome of such a study could be that particles unable to fuse at pH 6.7 would undergo fusion upon further acidification to pH 6.3. This would be in line with a recent study in DENV suggesting that flavivirus fusion within living cells is very efficient, with 1 out of 6 particles that bind to the cell eventually undergoing membrane fusion (van der Schaar et al., 2007).

Major rearrangements of the viral glycoproteins also play an important role in the assembly and maturation of flavivirus particles. The morphogenesis of flavivirus particles is initiated when newly formed nucleocapsids containing the viral genome bud into the endoplasmatic
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reticulum (ER) and are enclosed by a lipid bilayer anchoring the viral transmembrane proteins prM and E (Mackenzie & Westaway, 2001; Lorenz et al., 2002; Lindenbach & Rice, 2003). Within the resulting immature virions, the prM and E proteins are associated as heterodimers which in turn form 60 trimers that protrude from the virion surface, giving immature particles a “spiky” appearance (Zhang et al., 2003; Zhang et al., 2007). Analysis of the X-ray crystallographic structure of a recombinant DENV prM-E fusion protein has provided some insight into the mechanism by which the flavivirus particle is stabilized by the prM protein (Li et al., 2008). Like the fusion process, maturation too is highly regulated in a pH-dependent manner. Within the heterodimeric conformation, the M polypeptide portion of the prM protein is extended alongside DII of the E-protein, with the pr-portion covering the fusion loop at its distal end. The virus particles mature during transit through the Golgi apparatus. Upon exposure to the low pH conditions of the Trans Golgi network (TGN), the particle adopts a conformation in which the E-proteins are arranged as homodimers, analogous to the smooth prefusion conformation of the mature particle. This structural rearrangement allows for cleavage of the prM protein by the host endoprotease furin. Importantly, the pr-peptide remains associated with the fusion loop through electrostatic interactions until the particle is exposed to neutral pH (Yu et al., 2008). Dissociation of the pr-peptide after exocytosis primes the particle to undergo membrane fusion upon subsequent exposure to low pH. Indeed, in Chapter 3, we observed that fully immature WNV particles do not undergo membrane fusion with liposomes when exposed to pH values of 6.3 or lower. Furthermore, in line with the mechanism proposed by Li and coworkers (Li et al., 2008), in vitro maturation of immature virus by exposure to pH 6.0 in the presence of furin followed by back-neutralization, fully activated the fusogenic properties of immature WNV particles (Chapter 3, Fig. 3).

Interestingly, the flavivirus maturation process is not very efficient. We have shown that the protein composition of wildtype preparations of WNV or DENV grown on BHK-21 or C6/36 cells respectively, contains on average ~30% prM (Zybert et al., 2008; Chapter 3). In a recent study, an attempt was made to investigate the distribution of prM on a per particle basis using sequential immunoprecipitation (Junjhom et al., 2010). It was found that extracellular virus consists of three subpopulations of virus: fully mature (containing only M), fully immature (containing only prM) and partially immature (containing both M and prM). This experimental evidence is supported by cryo-electron microscopic (cryoEM) observations of particles that have a partially spiky and partially smooth appearance. Given our results in Chapter 3, the heterogeneous distribution of prM may be an important factor in controlling the fusogenic properties of a virus particle. It will be of interest to determine how many prM proteins per virus particle would still allow that virion to undergo membrane fusion.

ANTIBODY RESPONSE TO FLAVIVIRUS INFECTION

Analyses of patients serum samples and repertoire cloning of B-cells isolated from patients have provided insights into the polyclonal Ab response to flavivirus infections. These studies show that Abs raised in response to flavivirus infections are directed predominately against the E, prM and NS-1 proteins (Throsby et al., 2006; Lai et al., 2008; Dejnirattisai et al., 2010). Interestingly, 90 % of the E-specific response represent weakly neutralizing Abs and therefore it is
generally assumed that they do not significantly contribute to protection (Throsby et al., 2006; Oliphant et al., 2006; Oliphant et al., 2007; Lai et al., 2008; Crill et al., 2009; Dejnirattisai et al., 2010). Indeed, these cross-reactive Abs have been postulated to be involved in enhancement of flavivirus infection (Pierson et al., 2007; Cherrier et al., 2009), as will be discussed later. Potently neutralizing MAbs against WNV and DENV have been previously mapped to a highly exposed epitope within DIII and have been considered to be crucial for in vivo protection.

Other studies have shown that anti-prM Abs can also provide passive protection against DENV infection in mice (Kaufman et al., 1987; Falconar, 1999). It is unclear how Abs raised against prM might neutralize infection. Instead, they have recently been shown to enhance infection of immature DENV on FcR-bearing cells (Dejnirattisai et al., 2010; Rodenhuis-Zybert et al., 2010). Anti-NS1 Abs have been shown to protect by targeting NS-1 proteins expressed on the cell surface, thereby promoting the clearance of infected cells through FcR-dependent mechanisms (Chung et al., 2006).

**NEUTRALIZATION OF FLAVIVIRUS INFECTION: UNDERSTANDING THE ROLE AND FUNCTION OF ANTIBODIES**

The modus operandi of an Ab critically depends on its binding epitope and considerable effort has been made to identify regions on the flavivirus virion that elicit potently neutralizing Ab responses. More particularly, many studies have focused on mapping of the binding epitopes of Abs that efficiently cross-neutralize all four DENV serotypes in attempts to guide dengue vaccine development. Mapping of the binding sites of potently neutralizing Abs has revealed that neutralizing epitopes can be found throughout the E-glycoprotein (Crill & Roehrig, 2001; Morita et al., 2001; Goncalvez et al., 2004; Nybakken et al., 2005; Oliphant et al., 2006; Oliphant et al., 2007; Sukupolvi-Petty et al., 2007; Lai et al., 2007; Shrestha et al., 2010; Sukupolvi-Petty et al., 2010; Brien et al., 2010) and may even span the E-homodimer interface (Throsby et al., 2006; Chapter 5; Crill et al., 2009; Sukupolvi-Petty et al., 2010). In general, however, potently neutralizing Abs map to epitopes within DIII (Nybakken et al., 2005; Oliphant et al., 2006; Oliphant et al., 2007; Sukupolvi-Petty et al., 2007; Shrestha et al., 2010; Brien et al., 2010). X-ray crystallographic and yeast display epitope mapping studies have demonstrated that the potently neutralizing anti-WNV MAb E16 engages a highly accessible epitope on the lateral ridge of DIII (Nybakken et al., 2005). A structurally analogous epitope on the lateral ridge of DIII was later identified on the DENV E-glycoprotein (Shrestha et al., 2010; Brien et al., 2010; Wahala et al., 2010). Importantly, because of large sequence variation of residues within this epitope, distinct serotypes or genotypes are poorly neutralized by DIII lateral ridge MAbs. Intriguingly, compared to cross-reactive Abs, DIII Abs are present in much lower levels in human serum and several studies have now questioned their contribution to in vivo protection against WNV and DENV infections (Oliphant et al., 2007; Wahala et al., 2009). Recently, a number of studies have suggested the involvement of Abs binding to conformational epitopes such as the homodimer interface, in protection against flavivirus infection (Throsby et al., 2006; Chapter 5; Wahala et al., 2009; Sukupolvi-Petty et al., 2010). Abs recognizing conformational epitopes have been shown to comprise approximately 30% of the Ab response to WNV infection (Throsby et al., 2006), but are often overlooked in large IgG-screenings
because recombinant proteins instead of whole virions are used as antigen. In Chapter 5, we describe two highly neutralizing MAbs isolated from patients naturally infected with WNV, CR4354 and CR4348, that did not bind recombinant E in yeast-display mapping experiments, but readily bound whole virions. Importantly, binding was sensitive to exposure to low-pH, suggesting the involvement of a conformational epitope. Although CR4354 and CR4348 were shown to protect mice against lethal WNV challenge, the contribution of MAbs recognizing conformational epitopes to protection in humans remains to be determined. In contrast, more weakly neutralizing MAbs were found to be cross-reactive, recognizing more conserved epitopes predominantly on DI and DII (Oliphant et al., 2006; Stiasny et al., 2006; Lai et al., 2008). The large differences in neutralization potency between type-specific and cross-reactive MAbs have been related to the surface accessibility of their respective epitopes (Sukupolvi-Petty et al., 2007; Gromowski et al., 2008).

Role of occupancy in neutralization
Neutralization of flaviviruses is a ‘multi-hit’ phenomenon and occurs when the number of Abs that bind the virion reaches a required threshold (Pierson et al., 2007). The fraction of epitopes occupied by a MAb at a given concentration is a major determinant of the neutralizing potency of MAbs (Klasse & Sattentau, 2002). Many of the conserved epitopes recognized by cross-reactive MAbs are expected to be poorly exposed on the virion surface, limiting the number of sites available for engagement by MAbs. In addition, epitope availability may be limited further by the unconventional arrangement of E-dimer rafts on the virion. Within mature particles, the E-glycoprotein homodimers exist in three chemically distinct environments, resulting in differential surface accessibility of epitopes in each of these environments (Pierson et al., 2007). Together, these factors critically alter the number of binding sites available for occupation by MAbs. It was found that neutralization of WNV by MAbs recognizing the highly accessible DIII lateral ridge requires engagement of less than half of the available epitopes (Pierson et al., 2007). Conversely, many cross-reactive MAbs require occupancy levels exceeding 90% to reach the threshold for neutralization. The potency of MAbs that require high levels of occupancy for neutralization is strongly influenced by the maturation state of the virion (Nelson et al., 2008). It has been postulated that the presence of uncleaved prM within partially immature particles alters the availability of epitopes that are predicted to be occluded within the mature virion. Indeed, virions with higher levels of prM were shown to be more sensitive to neutralization in non-FcR expressing cells by cross-reactive MAbs than mature virions (Nelson et al., 2008).

Mechanisms of antibody neutralization: inhibition of membrane fusion
Abs are uniquely equipped to potently inhibit viral infection of target cells by directly binding to the virion. The textbook dogma states that Abs inhibit cellular entry by blocking interactions with the virus receptor. This view has proved somewhat outdated as Abs have been shown to be able to inhibit viral entry at many different stages including binding, internalization by endocytosis, membrane fusion, capsid uncoating and trafficking to the appropriate cellular location (reviewed by Klasse & Sattentau, 2002; Reading & Dimmock, 2007). In case of flaviviruses, MAbs have been described that specifically inhibit viral binding to non-Fc-receptor expres-
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sing cells (Roehrig et al., 1998; Hung et al., 1999; Crill & Roehrig, 2001). Other MAbs were found to neutralize not only cellular attachment but also at a postattachment stage. Gollins and Porterfield (Gollins & Porterfield, 1986a) were the first to show that Abs are able to neutralize viral infection by inhibiting fusion of the viral envelope with the endosomal membrane. Ab-mediated neutralization of membrane fusion has since been shown to be a common mechanism amongst flaviviruses (Gollins & Porterfield, 1986a; Randolph & Stollar, 1990; Roehrig et al., 1998; Butrapet et al., 1998; Crill & Roehrig, 2001; Goncalvez et al., 2004; Nybakken et al., 2005; Stiasny et al., 2007; Lai et al., 2007; Chapters 4 and 5).

In Chapter 4, we show that the highly neutralizing anti-WNV MAb E16 potently inhibits membrane fusion with liposomes. Intriguingly, cryo-EM reconstructions have revealed that E16 inhibits an intermediate step in the fusion process, thereby locking the virion structure in a dead-end conformation upon low-pH exposure (Kaufmann et al., 2009). The mechanism of inhibition proposed by Kaufmann and coworkers is in agreement with the observation that E16 neutralizes WNV at very low fractional occupancy (Pierson et al., 2007). Indeed, the low stoichiometric threshold may result from this ‘active’ mode of inhibition; trapping a virion in a dead-end conformation may require initial binding of a single Ab whereas inhibition of cellular attachment likely requires engagement of all available receptor-binding sites (Lok et al., 2008).

An important feature of fusion-inhibitory Abs is they may block viral infection irrespective of the mode of entry. Abs that exclusively neutralize infection through blockade of cellular attachment are not expected to block FcR-mediated entry of opsonized virus particles, possibly leading to enhancement of infection (as discussed in the next section). Regardless of the entry pathway, however, fusion with the endosomal membrane is an obligatory step in the flavivirus lifecycle and remains an important target for neutralization by fusion-inhibitory Abs.

Enhancement of infection may occur when the number of Abs engaging a virion is below the threshold required for neutralization. This threshold was shown to depend on epitope accessibility and Ab-affinity. Importantly, Abs that fail to meet the occupancy requirements for neutralization may still have bound in sufficient numbers to allow their opsonization into FcR-bearing cells. Ab-dependent enhancement of infection (ADE) was first described by Halstead and coworkers (Halstead & O’Rourke, 1977; Halstead et al., 1977) and involves the enhanced uptake of opsonized DENV particles into monocytes, macrophages and dendritic cells. The higher viral loads resulting from increased numbers of infected cells have been postulated to be crucial in the development of severe disease.

Influence of particle maturation status

It has become clear that the maturation status of the flavivirus particle greatly influences the availability of epitopes on the E-glycoprotein (Nelson et al., 2008; Cherrier et al., 2009) Regions on DI and DII that are occluded within the mature virion are expected to be more accessible within the context of immature virions. In fact, recent cryoEM analyses of the cross-reactive
MAb E53, targeting the fusion loop, in complex with wild-type or immature virions indicate that Fab-fragments of E53 fail to engage mature particles whereas they readily bind (partially) immature particles (Cherrier et al., 2009). As this MAb does not readily bind mature particles, it is unlikely to reach the occupancy requirements for neutralization. This has indeed been shown for E60, another cross-reactive MAb directed against the fusion loop. Several studies have demonstrated that E60 fails to fully neutralize infection, even at saturating concentrations (Oliphant et al., 2006; Pierson et al., 2007; Nelson et al., 2008). Interestingly, the presence of a virus fraction resistant to neutralization by E60 was also observed in fusion measurements with liposomes (Chapter 4). At the highest concentration tested, under conditions of MAb excess, approximately 30% of the virions within the population still underwent fusion. The apparent heterogeneity within the population likely relates to the maturation state of individual virions, although this warrants further investigation. If we assume that the fully mature virions within the population are not susceptible for neutralization by E60, and thus represent the resistant fraction of 30%, it would follow that the neutralized fraction would consist of particles more susceptible for neutralization: partially immature particles. The observations therefore suggest that partially immature particles are capable of fusion with liposomal membranes. The notion that partially immature particles are infectious is supported by Nelson and coworkers (Nelson et al., 2008). In their study, they conclude that particles containing higher levels of prM are more readily neutralized by cross-reactive MAbs, an observation implicitly implying that these particles are themselves infectious. Also, anti-prM MAbs have been shown to be neutralizing, albeit to a low extent, suggesting that binding of these MAbs to the prM portion of partially immature particles blocks their infectivity through mechanisms that remain to be defined (Kaufman et al., 1989; Vazquez et al., 2002; Dejnirattisai et al., 2010). The ability of cross-reactive Abs, which are highly abundant within polyclonal serum, to neutralize flavivirus infection is therefore highly dependent on the prM content of individual particles.

Influence of antibody affinity
Detailed epitope mapping of numerous MAbs raised in mice against WNV and DENV has revealed that Ab-binding epitopes consist of only a limited number of residues. Anti-WNV MAb E16 was found to engage a conformational epitope formed by four discontinuous strands on the lateral ridge of DIII (Nybakken et al., 2005). Yeast surface display epitope mapping identified four central residues that are critically involved in binding of E16. An analogous DENV MAb 3H5, also associated with strong neutralization, was mapped to a structurally identical region on the DIII lateral ridge of DENV-2 (Sukupolvi-Petty et al., 2007). Binding of this MAb was shown to rely on three central residues at two adjacent strands. The notion that binding of potently neutralizing MAbs depends on only a small number of residues has considerable consequences for our understanding of Ab neutralization. One of the main implications is that small changes within the epitope can result in large differences Ab-affinity, which critically influences binding specificity and neutralization capacity. This was recently exemplified by several studies investigating the ability of DIII lateral ridge MAbs to potently neutralize multiple DENV-1, DENV-2 and DENV-3 genotypes (Shrestha et al., 2010; Sukupolvi-Petty et al., 2010; Brien et al., 2010; Wåhala et al., 2010). Despite the high degree of sequence conserva-
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between these genotypes, large differences in neutralization potency and \textit{in vivo} protection were observed. These differences were caused by naturally occurring sequence variations within the epitope. In addition, Shrestha and coworkers postulated that genotype-specific variation in residues outside the binding epitope may contribute to altered neutralization potency (Shrestha \textit{et al.}, 2010). Indeed, changes in residues located outside an epitope have been shown to affect neutralization without abrogating binding of the MAb to its epitope (Goncalvez \textit{et al.}, 2004; \textbf{Chapter 5}). To conclude, affinity of binding is an important factor because it critically influences MAb engagement of virions. In heterologous DENV infections, MAbs are likely to bind poorly and cause enhancement of infection. Alternatively, low-affinity MAbs may be internalized along with the particle and might dissociate upon exposure to low-pH, allowing the virion to initiate infection.

\textit{Antibody-mediated uptake of immature flaviviruses}

The relevance of prM-containing flavivirus particles has been demonstrated by several recent papers. Importantly, Rodenhuis-Zybert and coworkers found that fully immature DENV particles opsonized with anti-prM MAbs are capable of infectious entry into FcR-bearing cells (Rodenhuis-Zybert \textit{et al.}, 2010). They observed that furin-cleavage upon endocytosis of opsonized particles was crucial for infectivity. This notion was extended to WNV in \textbf{Chapter 6}, where high infectious titers were recovered upon infection of murine macrophages with anti-prM opsonized immature WNV particles. Furthermore, infectious virus could be recovered from blood serum and brain of mice infected with fully immature WNV complexed with anti-prM MAb, while no virus was found when the mice received immature virus alone (\textbf{Chapter 6}).

Not only prM Abs but also E Abs are able to interact with prM-containing flavivirus particles. In \textbf{Chapter 7}, the entry pathway of fully immature DENV and WNV complexed with the cross-reactive anti-E MAb E53 was investigated. An important finding of this study is that E53 indeed supported the infectious entry of fully immature DENV and WNV. However, MAb E53 failed to promote the infectious properties of immature DENV on human leukemia K562 cells while it readily enhanced infectivity on murine P388D1 macrophages over a broad range of concentrations. It is demonstrated that opsonization of immature DENV with E53 shifts the optimum pH at which furin-mediated cleavage of prM (a required step in the entry of fully immature particles) takes place to lower pH values, likely explaining the differential enhancement in the two cell lines. It is not well understood what distinguishes DENV from WNV in this respect, although it is interesting to note that the E53 epitope on both viruses differs by one residue. This difference may cause considerable variation in binding affinity, either at neutral or low pH, allowing the MAb to dissociate from WNV but not DENV. The Ab-mediated blockade of furin cleavage of immature particles is a novel level of neutralization, relevant exclusively to the infectious entry of prM-containing virions and should be investigated in more detail.

Another mechanistical aspect of the entry of immature particles that remains to be clarified is how the pr-peptide dissociates upon furin-cleavage of the prM protein, rendering the particle fusogenic. According to current models of the flavivirus maturation process, the pr-peptide is released from the virion upon exposure to neutral pH (Yu \textit{et al.}, 2008). Paradoxically, the immature particle encounters increasingly lower pH values upon endosomal uptake and we
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propose that exposure to acidic pH may trigger the release of the pr-peptide. Functional studies suggest that pr-dissociation does not occur at pH levels as low as 5.5 (Yu et al., 2009). However, it is well known that phagosomes are acidified upon internalization of cargo and subsequently fuse to lysosomes (pH 5.0), making it likely that opsonized complexes are exposed to pH values below 5.5 (as reviewed by: Mellman et al., 1986). Indeed, recent preliminary observations of entry of opsonized immature DENV particles into living cells suggest that the time-to-fusion of these complexes is somewhat prolonged compared to non-opsonized wild type DENV (unpublished observations Ayala-Nunez, Wilschut, Smit). This observation would be in line with current models of acidification of endocytic vesicles where the initial acidification to pH 6 occurs within 5 minutes upon internalization, followed by a subsequent slow acidification to pH 5 within 30 - 40 minutes (Mellman et al., 1986). Analyses of the entry kinetics of opsonized immature particles, perhaps coupled with intra-endosomal pH measurements, are therefore expected to provide important insights into the functional mechanism of maturation upon entry.

Recent studies have implicated the involvement of prM-containing particles in the enhanced disease associated with secondary DENV infections through association with non-neutralizing MAbs (Dejnirattisai et al., 2010; Rodenhuis-Zybert et al., 2010; Schmidt, 2010). It is tempting to speculate that activation of the infectious properties of these otherwise non-infectious particles contributes to high viremia in secondary infections by increasing the number of infected cells. The identification of the functional aspects described above in this novel entry mechanism are of significant importance and may lead to new strategies to prevent the infectious uptake of partially immature particles.

PERSPECTIVES

Considerations for vaccine design
An important goal of flavivirus research has been to guide the development of safe and efficient vaccines against DENV. Recent advances in our understanding of the mechanisms of flavivirus neutralization and how failure to neutralize relates to enhancement of infection have revealed some important aspects that have not yet been considered in vaccine strategies. One of the major findings is the possible involvement of prM-containing DENV particles in disease development through FcR-dependent mechanisms. Abs directed against the prM protein were found to be highly cross-reactive and weakly neutralizing. It has therefore been suggested that DENV antigens used in the vaccine formulation should be adapted such that they do not readily give rise to these weakly neutralizing MAbs (Dejnirattisai et al., 2010). A vaccine consisting of merely fully mature virions may therefore meet these conditions.

Antibodies as antiviral therapeutics
The identification of potently neutralizing MAbs that provide robust protection against WNV in rodent challenge models has prompted researchers to explore their possible use as therapeutics in humans. As immunocompromised individuals are more likely to develop WNV encephalitis, a therapeutic should potently control viral spread and allow rapid clearance of viruses from the central nervous system. MAb E16 has been demonstrated to protect mice from lethal WNV challenge when administered as a single dose at 5 days postinfection. The application of
humanized E16 as a therapeutic against WNV is currently being investigated in clinical trials (Diamond, 2009). Interestingly, several MAbs were recently described to potently inhibit infection of influenza and HIV through blockade of membrane fusion (Ekiert et al., 2009; Alam et al., 2009). These MAbs were shown to bind a transient structural intermediate that is only exposed upon fusion. Importantly, the epitopes engaged by these MAbs are highly conserved because of their critical role in the fusion process. As a result, these MAbs are broadly cross-reactive, recognizing a wide range of strains and variants. It would be of interest to identify analogous fusion-inhibitory anti-flavivirus MAbs as they may represent a novel class of highly potent antiviral therapeutics with the ability to cross-neutralize multiple serotypes and genotypes.
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