The role of antibodies in controlling flavivirus cell entry
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Enhancing effect of a fusion-loop antibody on the infectious properties of immature flavivirus particles


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Chapter 7

ABSTRACT

Flavivirus-infected cells secrete a mixture of mature, partially immature, and fully immature particles into the extracellular space. Although mature virions are highly infectious, prM-containing fully immature virions are non-infectious largely because the prM protein inhibits the cell attachment and fusogenic properties of the virus. If, however, cell attachment and entry are facilitated by anti-prM antibodies, immature flavivirus virus become infectious after efficient processing of the prM protein by the endosomal protease furin. A recent study demonstrated that E53, a cross-reactive monoclonal antibody (MAb) that engages the highly conserved fusion loop peptide within the flavivirus envelope glycoprotein, preferentially binds to immature flavivirus particles. Here, we investigated the infectious potential of fully immature WNV and DENV particles opsonized with E53 MAb, and observed that like anti-prM antibodies, this anti-E antibody also has the capacity to render fully immature flavivirus particles infectious. E53-mediated enhancement of both immature WNV and DENV depended on efficient cell entry and the enzymatic activity of the endosomal furin. Furthermore, we also observed that E53-opsonized immature DENV but not WNV particles required a more acidic pH for efficient cleavage of prM by furin, adding greater complexity to the dynamics of antibody-mediated infection of immature flavivirus virions.
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INTRODUCTION

Flaviviruses, including dengue virus (DENV serotypes 1, 2, 3, and 4) and West Nile virus (WNV), are small, enveloped, positive-strand RNA viruses that are transmitted to humans primarily by arthropods. On the flavivirus surface there are 180 copies of two transmembrane proteins: the major (51–60 kDa) envelope glycoprotein E, and the smaller (8 kDa) membrane protein M. In the mature virion, the E glycoproteins are organized in 90 head-to-tail homodimers that lie flat on the viral surface. X-ray crystallography studies revealed that the ectodomain of each E monomer is comprised of three structural domains: DI, DII and DIII, connected by flexible hinges. The tip of DII contains a conserved region termed the “fusion loop”, which is required for the low-pH-driven membrane fusion of the viral membrane with the host endosomal membrane.

Assembly of flavivirus particles occurs at the endoplasmic reticulum (ER) by formation of immature virions. In immature particles, the E protein associates with prM, the precursor protein of M. The 90 E–prM heterodimers protrude from the viral envelope as 60 trimeric spikes. In this conformation, the pr peptide of the prM protein caps the fusion loop located at the distal end of each E monomer within the trimer. Maturation of flaviviruses occurs during transit through the secretory pathway. In the mildly acidic lumen of the trans-Golgi network (TGN), the viral envelope proteins undergo low-pH driven conformational changes including dissociation of the prM-E heterodimers and formation of E homodimers. Thereafter, the endoprotease furin cleaves the prM protein into a small M protein and a pr peptide. The pr peptide dissociates from the virion upon release of the particle to the extracellular milieu, which completes the formation of mature infectious virus.

The functional importance of flavivirus maturation has been investigated in significant detail. Multiple studies have shown that fully immature particles are non-infectious, with the presence of prM obstructing the low-pH-induced conformational changes in the viral E glycoprotein required for membrane fusion. These observations led to the hypothesis that prM acts as a chaperone preventing premature fusion of progeny virions in the acidic compartments of the secretory pathway. Indeed, in vitro studies have shown that fusogenic activity of immature particles could be restored upon furin treatment, demonstrating that cleavage of prM to M is required to render flavivirus particles infectious.

We recently observed that fully immature particles become significantly infectious when opsonized with anti-prM monoclonal or serum antibodies. The prM antibodies facilitated efficient binding and entry of immature DENV into cells expressing Fcγ-receptors. Furthermore, furin activity within the target cell was required to render immature particles infectious indicating that immature particles undergo maturation after cell entry. The ability of prM antibodies to rescue infectious properties of immature DENV was recently corroborated by observations of Dejnirattisai et al., using human MAbs.

In addition to antibodies against prM antibodies, those recognizing the E protein also can bind to immature virus particles. E53 is a fusion-loop-specific inhibitory anti-E MAb that preferentially binds to the immature form of WNV and DENV particles. Consistent with this, E53 and other fusion-loop-specific MAbs neutralized partially mature (prM-containing) but not fully mature (prM-absent) WNV virions. X-ray crystallographic analysis of E53 Fab...
fragments complexed to WNV E protein have revealed that E53 engages 12 residues within the fusion peptide (G104, C105, G106, L107, G109, K110) and adjacent b-c loop (C74, P75, T76, M77, G78, E79) of DII. Fitting of the E53 Fab-WNV E crystal structure onto the cryo-EM structure of immature virions suggested that E53 may neutralize infection by impeding the transition from immature to mature virus by steric hindrance.

In this study, we investigated the influence of the E53 MAb on infectivity of fully immature DENV and WNV particles. Surprisingly, we observed that E53 significantly enhances the infectious properties of immature WNV particles. For immature DENV, enhancement of infection was observed in a cell type dependent manner. Whereas in Fc-receptor-expressing human erythroleukemic K562 cells no infectivity was observed, a marked increase in viral infectivity was seen in murine macrophage-like P388D1 cells. Analysis of the internalization pathway of E53 opsonized immature DENV particles suggested that this is related to a more acidic pH threshold for furin cleavage that is required to occur within endosomal compartments of the target cells. Furthermore, we show that in human peripheral blood mononuclear cells E53 mediated enhancement of wt DENV preparation is primarily dependent on the activity of furin. Overall, this report shows for the first time that in addition to anti-prM antibodies, those against E protein also can render immature flavivirus particles infectious.

MATERIALS AND METHODS

Cell culture.
C6/36 Aedes albopictus cells were maintained in minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 25 mM HEPES, 7.5% sodium bicarbonate, penicillin (100 U/ml), streptomycin (100 μg/ml), 200 mM glutamine and 100 μM nonessential amino acids at 28°C, 5% CO2. Baby hamster kidney-21 (BHK21) cells were cultured in DMEM (Invitrogen), supplemented with 5% FBS, 10% tryptose phosphate broth, 25 mM HEPES, 7.5% sodium bicarbonate, penicillin (100 U/ml), streptomycin (100 μg/ml) and 200 mM glutamine at 37°C, 5% CO2. BHK21 clone 15 cells (BHK21-15) were maintained in DMEM (Invitrogen), containing 10% FBS, 25 mM HEPES, 7.5% sodium bicarbonate, penicillin (100 U/ml), streptomycin (100 μg/ml), 10 mM HEPES and 200 mM glutamine. Human adenocarcinoma LoVo cells were cultured in Ham’s medium (Invitrogen) supplemented with 20% FBS at 37°C, 5% CO2. Human erythroleukemic K562 cells were maintained in DMEM containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37°C, 5% CO2. Mouse macrophage-like P388D1 cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml), sodium bicarbonate (Invitrogen,7.5% solution) and 1.0 mM sodium pyruvate (GIBCO) at 37°C, 5% CO2. Human peripheral blood mononuclear cells (PBMCs) were maintained in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). PBMCs were isolated from heparinized blood samples collected from healthy persons using standard density centrifugation procedures with Lymphoprep™ (AXIS-SHIELD). The PBMCs were used immediately after isolation or cryopreserved at -150°C. On the day of infection, the percentage of CD14+, CD19 population within isolated PBMCs was determined (5% -10% depending on the blood donor) using cell surface markers CD-14 -FITC and CD19-R-PE purchased from commercial source (IQ Products).

Virus propagation.
DENV-2 strain 16681 was propagated in C6/36 cells as described previously. WNV strain NY 385-99 (generous gift of Dr. J. Goudsmit, Crucell B.V., Leiden, The Netherlands) was propagated after inoculation of BHK21 cells at an MOI of 0.1. Culture medium was harvested at 48 hpi, cleared of cellular debris, aliquotted, and stored at -80°C. Fully immature DENV and WNV preparations were generated in LoVo cells as described previously. [35S] methionine-labeled immature virus preparations of WNV and DENV were prepared as described. All virus preparations were analyzed with respect to the number of infectious (PFU) and genome-containing particles (GCPs) by plaque assay and quantitative (q)RT-PCR, respectively. For WNV, qRT-PCR analysis was performed using the
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forward primer 5'-GTT GGC GGC TGT TTT CTT TC-3', the reverse primer 5'-GGG ATC TCC CAG AGC AGA ATT-3' and a TaqMan probe 5'-FAM-AAT GGC TTA TCA CGA TGC CCG CC-TAMRA-3' (Eurogentec, Seraing, Belgium). DNA was amplified for 40 cycles (15 s at 95°C and 60 s at 60°C) on a StepOne real-time PCR instrument (Applied Biosystems, Carlsbad, CA) and the number of copies of WNV RNA was quantified using a standard curve based on a cDNA plasmid containing the non-structural genes of WNV NY99 (kind gift from Dr. G.P. Pijlman, Wageningen University, The Netherlands).

ELISA.
The reactivity of MAb E53 to immature DENV or WNV was determined by standard three-layer ELISA with a horseradish peroxidase-based detection system as described previously. In the experiments, wherein the effect of the low pH on the binding of E53 to immature virions was evaluated, additional 15-min acidic (pH 5.0, 5.5, 6.0, 6.5), washes were introduced.

Infectivity assays.
Virus or preformed virus-MAb complexes were incubated with K562 or P388D1 (2 x 10^5) cells at a multiplicity of 10, 100 or 1000 GCPs (MOG) per well for WNV and DENV, respectively. At 26 (WNV) or 43 (DENV) hpi, medium was harvested and virus yield was analyzed by plaque assay on BHK21-15 cells, as described previously. Virus-MAb complexes were formed by incubating virus for 1 h at 37°C with increasing concentrations of MAB E53 in cell culture medium containing 2% FBS prior to the addition to cells. The DENV anti-prM mAb 70-21 was included as a positive control. In furin blockade experiments, the cells were treated with 25 μM of the furin-specific inhibitor decanoyl-L-arginyl-L-valyl-L-lysyl-L-arginyl-chloromethylketone (decRRVKR-CMK, Calbiochem, Darmstadt, Germany) prior to and during infection.

Binding and cell internalization assays.
To determine the number of bound/internalized viruses per cell, virus or virus-MAb complexes were incubated with K562 (2 x 10^5) cells at MOG 1000 for 1 h at 37°C as described previously. Subsequently, cells were washed extensively with PBS containing MgCl₂ and CaCl₂ to remove unbound virus-MAb complexes. To quantify internalized virions, cells were treated for 1.5 h with 0.5mg of proteinase K (Invitrogen). Viral RNA was extracted from cells and from control cells' washes using the QIAamp Viral RNA mini kit (QIAGEN, Valencia, CA). cDNA was synthesized from the viral RNA with reverse-transcription PCR (RT-PCR), and copies were quantified using qRT-PCR analysis.

In vitro furin cleavage assay.
[^35S]methionine-labeled immature particles or viral immune-complexes were incubated with furin (New England Biolabs, Ipswich, MA) for 16 h at pH 6.0, as described previously or at a specified pH as indicated in the Results. Following furin treatment, viral proteins were visualized by subjecting the samples to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and phosphorimaging analysis on a Cyclone scanner (Perkin Elmer, Waltham, MA).

RESULTS

E53 renders immature WNV and DENV particles infectious in a cell-dependent manner.
The infectious properties of immature WNV and DENV particles opsonized with increasing amounts of the fusion loop cross-reactive MAb E53 were investigated in two Fcγ-receptor-expressing cell lines, human leukemia K562 cells and murine P388D1 macrophages. To this end, we first generated immature WNV and DENV particles in furin-deficient LoVo cells using a published protocol. The specific infectivity of the LoVo cell-derived virus used in this study was reduced greater than 10,000-fold compared to that of the st virus preparation, in agreement with our previous data. After these initial characterizations, K562 and P388D1 cells were infected with LoVo cell-derived WNV and DENV particles in the presence of increasing concentrations of E53. At 26 (WNV) or 43 (DENV) hours post-infection (hpi), the (infectious) viral titer in the supernatant was determined by plaque assay. Interestingly,
coating of immature WNV particles with the E53 MAb significantly stimulated viral infectivity in both K562 and P388D1 cells (Fig. 1A and B, \( P < 0.0001 \)). At an E53 concentration of 0.012 and 0.12 μg/ml in K562 cells and 0.12 μg/ml in P388D1 cells greater than a 1,000 fold enhancement of infectious WNV production was observed. At higher MAb concentrations, neutralization of infection was seen. An E53 antibody concentration of 0.12 μg/ml corresponds to 8 x 10^{-10} M and addition of 2 x 10^4 MAb molecules per virion, indicating that a large excess of antibody is required to trigger infectivity. Surprisingly, whereas E53 did promote infectivity of immature DENV in P388D1 cells (Fig. 1D, \( P < 0.0001 \)), over a broad range of antibody concentrations, no infectivity was observed in K562 cells, while the anti-prM mAb 70-21 did stimulate infectivity of immature DENV in these cells (Fig. 1C, ref. 23).

**Figure 1.** E53 renders immature WNV and DENV particles infectious in cell dependent manner.

Immature WNV (A, B) and DENV (C, D) particles were incubated with increasing concentrations of E53 for 1 h at 37°C. For DENV, the prM antibody 70-21 (40 ng/ml) was included as a positive control. K562 cells were infected at MOI 10 and 100 for WNV and DENV, respectively. At 26 (WNV) and 43 (DENV) hpi, virus production was measured by plaque assay on BHK21-15 cells. Data are expressed as the mean of at least three independent experiments. Error bars represent standard deviations (SD); (n.d.) denotes “not detectable”.

The lack of infectivity of E53-opsonized immature DENV in K562 cells is not caused by impaired virus internalization.

The observed difference in the enhancing effect of E53 in K562 vs. P338D1 cells between WNV and DENV prompted us to investigate the entry of immature flavivirus particles in these...
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While the observation showing that E53-opsonized immature WNV is infectious in K562 cells suggests that E53 facilitates efficient entry of immature flavivirus particles into K562 cells, we assessed whether this was also true for immature DENV virions. Immature DENV and WNV particles were pre-incubated with increasing concentrations of E53 MAb and added to K562 cells for 1 hr at 37°C to allow cell binding and internalization. After extensive washing, the number of bound or, after treatment with proteinase K, internalized virions per cell was determined by quantitative RT-PCR. To reliably determine the number of bound GCPs per cell, the amount of virus added per cell was increased 10-fold compared to the concentration used in the infectivity experiments. Independent experiments showed that the higher concentration of input virus did not affect viral infectivity of immature virus by E53 (data not shown).

As expected, in the absence of E53 MAb or in the presence of control MAb, virtually no cell binding was observed for immature WNV or DENV, confirming that immature particles fail to interact efficiently with K562 cells (ref. 23 and Fig. 2). Notably, E53 facilitated efficient binding and cell entry of not only immature WNV but also immature DENV, demonstrating that the lack of infectivity observed for E53-opsonized immature DENV is not related to inefficient internalization to K562 cells.

Furin protease activity is required to render immature flavivirus particles infectious.

Given the binding and internalization findings in K562 cells, we next assessed the role of furin during cell entry as its protease activity in cells is crucial for rendering immature DENV particles opsonized by prM antibodies infectious. We treated K562 cells and P388D1 cells prior to and during infection with the furin inhibitor, decanoyl-L-arginyl-L-valyl-L-lysyl-L-arginyl-chloromethylketone (decRRVKR-CMK). The half-life of decRRVKR-CMK is 4 to 8 h. Indeed, we previously reported that this treatment does not interfere with the formation of infectious particles following infection with st DENV. Furin inhibitor was observed to only affect virus particle maturation upon addition of the compound at the moment of virion assembly. As shown in Figure 3, addition of furin inhibitor at the time of infection does not influence ma-

Figure 2. E53 facilitates efficient binding and cell entry of immature WNV and DENV.
E53-coated immature DENV (A) and WNV (B) particles were incubated with K562 cells at MOI 1,000 for 1 h at 37°C. Unbound virus was washed away and virus associated with K562 cells was detected by qRT-PCR analysis. Internalization was assessed after removal of the bound virus with proteinase K treatment. Data are expressed as the mean of at least three independent experiments performed in duplicates. Error bars represent standard deviations (SD); (n.d.) denotes “not detectable”.

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turation of newly assembled virions within infected cells. Importantly, the infectivity of E53-opsonized immature WNV (Fig. 3A and 3B for K562 and P388D1 respectively) and DENV particles in PD388D1 (Fig. 3C) was lost in the presence of the inhibitor, and thus, required the enzymatic activity of furin. These results confirm that furin cleavage of prM to M is a prerequisite step in the cell entry process of antibody-coated immature flavivirus particles, regardless of whether the enhancing antibody is directed at the prM or E proteins.

**E53 affects the cleavage of DENV prM by furin**

Because recent structural analysis suggested that E53 neutralizes infection by impeding the transition from immature to mature virus\(^3\), we hypothesized that the lack of infectivity of E53-bound immature DENV particles in K562 cells was related to the inability of furin to cleave prM to M, possibly due to steric hindrance. To evaluate this, we incubated \(^{35}\)S-methionine-labeled immature WNV and DENV in the absence and presence of E53 MAb with exogenous furin at pH 6.0, the condition that mimics the mildly acidic milieu of the early endosomal lumen. Subsequently, the protein composition of the virus particles was analyzed by SDS–PAGE and phosphorimaging. Whereas efficient cleavage of prM to M was observed for WNV particles in the presence of E53 and furin (Fig 4A), prM processing was completely inhibited under the same conditions for immature DENV particles (Fig. 4B). The ability of furin to cleave E53-
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While blockade of furin cleavage by E53 antibody explained the lack of enhancement of immature DENV in K562 cells, it was not consistent with the observed infectivity of antibody-bound immature DENV in P388D1 cells. Because endosomal pH can vary substantially between different cells \(^2\), flaviviruses undergo low-pH-triggered structural changes prompting dissociation of prM/E heterodimers, we hypothesized that E53 might bind to virus particles in a manner that impairs furin cleavage at mildly acidic pH, yet allowing furin cleavage at lower pH values. Initially, we attempted to examine the cleavage status of the virus after cell
entry in K562 cells but due to the low number of infected K562 cells even at high multiplicities of infection this could not be defined. As a surrogate model, we performed the same *in vitro* furin cleavage experiment as described above but now under different pH conditions. Immature DENV was incubated with HNE buffer containing E53 antibody or with the buffer alone and subsequently subjected to the furin cleavage experiment at pH 6.0; 6.0; 5.5, 5.0. Remarkably, E53 shifted the pH threshold for furin cleavage of DENV immature particles to more acidic values, indicating that antibodies can directly modulate the pH-dependent structural changes after entry (Fig. 4C).

Based on these results we hypothesized that E53 stays associated with immature DENV at mildly acid pH thereby preventing furin cleavage and infection and dissociates from the virus at lower pH values allowing furin cleavage and subsequent infection. To test this, we performed ELISA experiments in which low pH (range 6.5 to 5.0) washes were performed following the incubation with E53. Consistent with prior studies \(^3,^{21}\), we observed that E53 efficiently binds to immature WNV and DENV particles at neutral pH. In contrast to our hypothesis, we found that E53 binding to immature particles is not pH-dependent (Fig. 4D), which suggests that dissociation of E53 from the immature virion is not a prerequisite for furin cleavage. We now therefore propose, although difficult to substantiate, that exposure of E53-opsonized DENV to lower pH values is necessary to overcome the energy threshold that is required to induce the global rearrangement of the virion prior to furin cleavage.
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Furin activity is important for E53-mediated enhancement of the standard flavivirus preparations

Standard flavivirus preparations contain a mixture of mature, immature and partially mature virions, the latter containing a mixture of prM and M. Indeed, a substantial fraction of DENV particles secreted from C6/36 insect cells are partially mature. Since furin-activity is crucial to render fully immature virions infectious, we investigated whether E53, which preferentially binds to the immature virions, enhances infectivity of st virus preparations in a furin-dependent manner. We performed the enhancement assays in K562 cells (Fig. 6A/D), P388D1 cells (Fig. 5B/E) as well as in human PBMCs (Fig. 5C/F). Notably, E53 enhanced the infectivity of both st WNV preparations (Fig. 5A-C) and DENV (Fig. 5D-F). The level of enhancement for st WNV in K562 cells (2- to 5-fold, P < 0.05) and PBMCs (2-to 5-fold, P<0.05) was slightly less than in P388D1 cells (4- to 10-fold, P < 0.001). Interestingly, E53 also enhanced infection of st DENV in K562 cells (2- to 3-fold, P<0.05, Fig. 5D) albeit to a lesser extent than in P388D1 cells (70- to 200-fold, P< 0.001, Fig. 5E) or PBMCs (10- to 20-fold, P < 0.001, Fig. 5F). The enhanced infectivity of E53-opsonized st DENV in K562 cells was not dependent on furin activity, in agreement with the observation that E53-opsonized immature virions were not infectious in these cells. In comparison, the higher degree of furin-dependent enhancement seen for E53-opsonized st DENV compared to WNV in P388D1 and PBMCs cells suggests that DENV produced in insect cells contains a higher fraction of partially immature and fully immature particles (those requiring maturation upon entry) than WNV. This may also explain why a higher level of enhancement was seen in P388D1 cells and PBMCs compared to K562 cells, since furin cleavage of E53-opsonized immature DENV is likely inhibited in K562 cells.

DISCUSSION

In this study, we show for the first time that the flavivirus cross-reactive MAb E53, which maps primarily to the fusion-loop in DII, can render fully immature flavivirus particles infectious. Enhancement of infection by E53-opsonized immature DENV was cell type-specific since E53 stimulated infectivity of immature DENV particles in a murine macrophage cell line, but did not enhance infection in human K562 cells. E53 facilitated efficient binding and cell entry of both immature DENV and WNV in K562 cells, and E53-mediated enhancement of infection of immature virions was strictly dependent on furin activity in the target cell. Analysis of the pH-dependent furin cleavage step revealed that E53 inhibits prM cleavage of immature DENV particles at a mildly acidic pH values, but that this can be overcome at lower pH. This suggests that the ability of antibodies to stimulate infectivity of immature DENV is cell type-dependent, presumably due to the unique host environment, including the pH of endosomes, after virus entry. The prerequisite of furin activity for E53-mediated enhancement of wt DENV, but not that of immature WNV, not only substantiates the difference between these two flaviviruses, but also underlines the potentially important role of immature or partially mature DENV in antibody-dependent enhancement of infection.

The observation that anti-E antibodies can render immature flavivirus particles infectious is novel and has implications for our understanding of the mechanisms of virus cell entry. Within
Acidified endosomes, the immature virion undergoes a major structural rearrangement, allowing furin to cleave prM to M and a pr peptide. Recent data suggest that the pr peptide remains associated with the particle at pH 5.5. The pr peptide is believed to protect newly assembled virions from adventitious fusion during transit through the acidic trans-Golgi network, and to be released only after the particle reaches the extracellular milieu, which has a slightly basic pH. However, it remains unknown how the pr peptide is released after furin cleavage of immature particles within endosomes. We have previously hypothesized that following furin cleavage the pr peptide will be released from the particle due to its interaction with prM antibodies thereby enabling the E proteins to undergo the conformational change required for fusion. Here, we show that an anti-E MAb also stimulates the infectivity of immature virions. Thus, it seems more plausible that dissociation of the pr peptide from the virion can be triggered directly by specific conditions in late endosomes, such as e.g. the lower pH (~5.0) environment. This notion is supported by a recent study demonstrating that inhibition of fusion by pr peptide is less efficient in this lower pH range. Alternatively, cleaved immature particles may be recycled with or without antibody back to the plasma membrane and/or extracellular space to allow pr dissociation and subsequent initiation of infection upon re-entry of the virions into the endocytic pathway.

Previous structural studies have suggested that the fusion-loop MAb E53 may block the transition of immature to mature particles by steric hindrance. The results presented here confirm that E53 does inhibit cleavage of prM to M of fully immature DENV particles under mildly acidic pH values. By contrast, E53-opsonized immature WNV particles were processed efficiently under the same conditions. These data may explain, at least in part, a prior observation that fusion loop-specific MAbs that were generated against WNV (e.g., MAbs E18, E53, or E60) had far greater neutralizing activity against DENV infection and why they blocked WNV infection primarily at a stage of viral attachment rather than fusion as seen with DENV. Interestingly the neutralizing effect of E53 on the immature DENV particle was pH-dependent and cell type-specific, suggesting that depending on the cellular context, opsonized immature dengue particles may be neutralized or rescued. Thus, for DENV, the relative pH in the early endosomes presumably controls the fate of E53-opsonized immature virus. On the other hand, in K562 cells, which are reported to have a low early endosomal pH, immature DENV is neutralized, suggesting that optimum pH for furin cleavage is not the sole prerequisite for the immature virus to gain infectivity. From a structural perspective, it remains unclear why E53 has a distinct effect on immature WNV and DENV particles. Because the one amino acid difference (residue E77: DENV Q, WNV M) in the E53 structural epitope on WNV and DENV represents a polar amino acid, it is tempting to speculate that, at mildly acidic pH, E53 stabilizes the viral spike complex of immature DENV to such an extent that the global conformational changes are blocked whereas in case of immature WNV E53 does not prevent the conformational change and furin cleavage to occur.

The humoral response generally has a crucial function in controlling flavivirus infections. However, for DENV, antibodies are not only involved in viral clearance, but under certain conditions may also be associated with development of severe disease symptoms by so-called antibody-mediated enhancement (ADE) of infection. The ADE hypothesis suggests that at
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sub-neutralizing concentrations antibodies will target virions to Fcγ-receptor-bearing cells and, thus, expand the number of infected cells and consequently the viral load. Recent studies suggest that the ability of an antibody to neutralize or enhance infection may be modulated by the maturation state of a virus particle. Specifically, antibodies against prM and, as demonstrated in the present study, the fusion-loop on E can render non-infectious fully immature particles infectious. The hallmark of these antibodies is that they are generally cross-reactive between DENV serotypes and poorly neutralizing. In addition, these antibodies bind avidly to immature or partially mature virions, and thus may target these virus particles efficiently to Fcγ-receptor-bearing cells, underlining the potentially important role of immature or partially mature DENV in ADE and the pathogenesis of severe disease. Accordingly, the presence of (partially) immature virions and anti-E or anti-prM antibodies, which preferentially or solely interact with the immature aspect of these virus particles, may well be of particular importance for immune enhancement during secondary heterosubtypic DENV infection.

Based on our prior studies with anti-prM antibodies and those described here, we suggest that not only efficient entry but also particle maturation within the target cell represent important steps in antibody-mediated enhancement of DENV infection. Clearly, in the case of fully immature virus, furin-mediated maturation within the target cell is essential for rescue of viral infectivity. On the other hand, for st virus, which in addition to fully mature and immature virus also contains partially mature particles, furin cleavage is not essential since the mature aspect of these partially mature virions may undergo the low-pH-induced conformational change without additional cleavage of prM and release of pr peptide. Consistent with this model, E53 did stimulate the infectivity of st DENV in K562 cells, while these cells do not support furin cleavage of prM. However, in P388D1 cells, which do support viors maturation, E53-dependent enhancement of st DENV infection was much more pronounced than in K562 cells, indicating that cleavage of prM within the target cell significantly contributes to the enhancement of infection. Also, in human PBMC, which comprise major DENV target cells, E53-mediated enhancement of st DENV infection was strongly dependent on furin activity, again indicating that furin-mediated maturation, also in these physiologically important cells, is an important factor in antibody-mediated enhancement of infection.

Not only antibody-mediated enhancement, but also antibody neutralization of infection depends on the maturation status of the virion. Maturation of WNV particles reduced the ability of fusion-loop antibodies, such as E53, to neutralize infection. Given that fusion-loop MAbs preferentially recognize immature particles the decreased neutralizing activity of E53 with fully mature virions has been explained by the hypothesis that binding does not reach an occupancy sufficient for neutralization. Consistent with this notion, addition of the complement component C1q, which reduces the stoichiometric threshold of antibody neutralization, allowed E53 to neutralize fully mature WNV more efficiently. Based on these prior studies and the data presented here, it is now clear that the structural architecture of individual viral particles influences the outcome of infection. Future studies that define more precisely the biochemical and cell-biological mechanisms of antibody-mediated neutralization or enhancement of immature virus particles will undoubtedly clarify flavivirus disease pathogenesis and promote efforts for safe and effective vaccine development.
Chapter 7

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Infectious properties of E53-opsonized immature flaviviruses

REFERENCES


Chapter 7


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