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Review

# Antibody-mediated neutralization of flaviviruses: A reductionist view

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ABSTRACT

Flaviviruses are a group of ~70 small RNA viruses responsible for significant morbidity and mortality across the globe. Efforts to develop effective vaccines for several clinically important flaviviruses are underway. Antibodies are a significant component of the host's protective response against flavivirus infection with the potential to contribute to immunity via several distinct mechanisms, including an ability to directly neutralize virus infection. Conversely, virus-reactive antibodies have been implicated in the increased risk of severe clinical manifestations following secondary dengue virus infection. In this review, we will discuss recent progress toward understanding the molecular basis of antibody-mediated neutralization of flaviviruses. Neutralization requires engagement of the virion with a stoichiometry that exceeds a required threshold. From this perspective, we will discuss viral and host factors that impact the number of antibody molecules bound to the virus particle and significantly modulate the potency of neutralizing antibodies.



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## Introduction

Antibodies are glycoproteins that equip the adaptive humoral immune system with the capacity to recognize a constantly evolving spectrum of pathogens. A significant role for antibodies in protection of the host from virus infection has been established (reviewed in Parren and Burton, 2001). Antibodies exert their antiviral effects by virtue of a capacity to bind viruses and directly neutralize infectivity, as well as via effector functions coordinated by the crystallizable fragment (Fc) region of the antibody heavy chain (Burton, 2002; Nimmerjahn and Ravetch, 2008). Passive transfer of virus-reactive antibody or immune sera has been shown to confer protection from infection by many virus types (reviewed in Parren and Burton, 2001; Roehrig et al., 2001; Sawyer, 2000). These findings highlight the general importance of humoral immunity to virus infection, and may

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enable the identification of desirable functional properties of the antibody response to be targeted by vaccination.

Flaviviruses are a group of positive-stranded RNA viruses capable of significant morbidity and mortality that are transmitted to humans primarily by arthropod vectors (Lindenbach et al., 2007). Highly-effective vaccines for several flaviviruses are in use, including yellow fever virus (YFV) (Barrett and Teuwen, 2009), tick-borne encephalitis virus (TBEV) (Heinz et al., 2007), and Japanese encephalitis virus (JEV) (Halstead and Thomas, 2010). A vaccine to protect against dengue virus (DENV) is not yet available despite an urgent need (>50 million human infections annually) and considerable effort by the public and private sectors (Guy et al., 2010; Whitehead et al., 2007). DENV vaccine development is complicated considerably by a perceived need to simultaneously protect against the four serotypes of DENV that circulate in nature, and the possibility that a non-protective humoral response may contribute to more severe clinical outcomes in vaccinated populations (Whitehead et al., 2007). Flavivirus-reactive antibodies may be protective or pathogenic depending on circumstances and context (Halstead, 2003). In this regard, understanding the breadth, specificity, and functional properties of the polyclonal antibody response to infection and vaccination will contribute significantly to the development of safe vaccines against flaviviruses.

A reductionist approach toward understanding how antibodies contribute to protection from flaviviruses considers how their neutralizing activity is controlled at the level of binding to individual virions and in what numbers (Burton et al., 2001; Della-Porta and Westaway, 1978). This stoichiometric viewpoint serves as an interesting reference from which to consider the impact of cellular factors and viral dynamics on antibody-mediated neutralization.

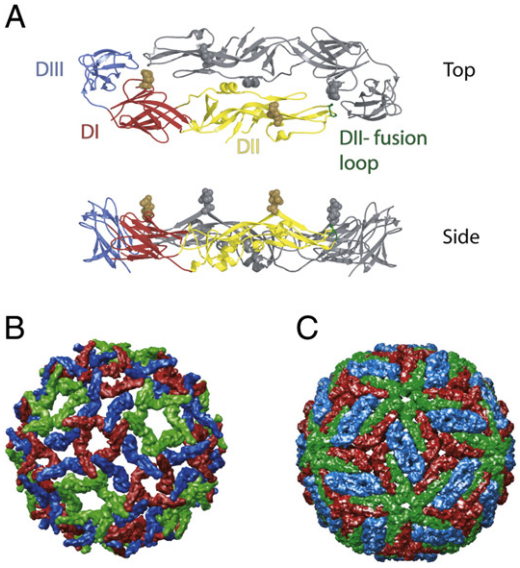


**Fig. 1.** Structure of the flavivirus E protein and its arrangement on the virion. A) Ribbon diagram of the anti-parallel flavivirus E protein dimer as seen from the top and side. Individual domains of the E protein are indicated, as is the fusion loop at the distal end of E-DII. The N-linked carbohydrate modifications on E are shown as brown spheres. B) Cryoelectron reconstruction of the flavivirus immature virion. Image was created using Chimera (http://www.cgl.ucsf.edu/chimera/). C) Cryoelectron reconstruction of the flavivirus mature virion illustrating the arrangement of E proteins in a T = 3 pseudo-icosahedron. Individual E proteins are colored according to their proximity to the 2-, 3-, or 5-fold symmetry axes (blue, red, and green, respectively).
Image obtained from the VIPERdb Virus Particle Explorer (http://viperdb.scripps.edu/) (Shepherd et al., 2006).

## Flaviviruses are complex antigens

Flaviviruses are small spherical virus particles composed of three structural proteins (capsid, envelope, and pre-membrane/membrane), an RNA genome, and a lipid envelope (Mukhopadhyay et al., 2005). The envelope (E) protein is a type II viral fusion protein composed of three distinct domains connected to the viral membrane by a helical structure called the stem anchor (Fig. 1A) (reviewed in Harrison, 2008). The E protein orchestrates the processes of virus assembly, budding, and entry into target cells. This protein is also the primary target of neutralizing antibodies; all three E protein domains contain epitopes recognized by antibodies capable of neutralizing infection under some circumstances (reviewed in Roehrig, 2003). Flaviviruses bud into the endoplasmic reticulum as immature virions on which the E proteins interact with the pre-membrane (prM) protein as heterotrimeric spikes arranged with icosahedral symmetry (Fig. 1B) (Zhang et al., 2003; Zhang et al., 2007). Interactions between the prM and E proteins prevent fusion of the virus with membranes of the producer cell during egress through acidic compartments of the secretory pathway (Guirakhoo et al., 1991; Heinz et al., 1994). Cleavage of prM by the cellular protease furin during transit through the Golgi network is a required step in the viral lifecycle that defines the transition from an immature non-infectious virus particle into an infectious form (Elshuber et al., 2003). The products of cleavage are a ~8 kDa M protein that remains associated with the virion, and a "pr" portion that disassociates from the virus particle upon release from the cell (Li et al., 2008; Yu et al., 2008), although as discussed below in greater detail, this cleavage event is often inefficient and results in the release of infectious virions that retain some uncleaved prM molecules. While prM-specific antibodies display limited neutralizing activity (Aaskov et al., 1988; Beltramello et al., 2010; Kaufman et al., 1989; Vazquez et al., 2002), it has been suggested that they contribute to the pathogenesis of DENV virus infection (Dejnirattisai et al., 2010; Rodenhuis-Zybert et al., 2010).

Mature flaviviruses incorporate 180 copies of the E protein arranged as anti-parallel dimers in an unusual herringbone pseudo-icosahedral symmetry pattern (Kuhn et al., 2002; Mukhopadhyay et al., 2003). In this configuration the E protein exists in three distinct chemical environments defined by proximity to the three different symmetry axes of the virion (Fig. 1C). This dense arrangement of E proteins adds considerably to the complexity of the antigenic surface of the virion as epitopes may be differentially accessible for antibody binding depending on their position on the virus particle (Kaufmann et al., 2006; Kaufmann et al., 2010; Nybakken et al., 2005).

## Models of the stoichiometric requirements for antibody-mediated neutralization

Considering how the number of antibody molecules bound to the virion determines whether or not virus neutralization occurs is a useful framework to explore factors that govern antibody potency. Two models to explain the behavior of neutralizing antibodies have been proposed and debated extensively. An excellent historical perspective of both schools of thought is provided by Parren and Burton (2001).

"Single-hit" concepts of neutralization postulate that neutralization occurs when the virion is bound by antibody at a "critical site" (Dulbecco et al., 1956). This model posits that antibody binding to functionally important locations on the virion is sufficient to disrupt required steps of the virus entry process and irreversibly inactivate virions (Mandel, 1976). In this regard, not all binding events are equivalent and antibodies bound to non-critical sites on the virion do not contribute to neutralization. Experiments demonstrating that neutralization proceeds via first order kinetics have been used to support this perspective, and suggest neutralization begins immediately after exposure to antibody (Dulbecco et al., 1956). However, interpretation of these results is somewhat controversial. The inability to observe a lag phase in kinetic neutralization experiments, corresponding to the time required to load virions with multiple antibody molecules, may simply reflect limitations of experimental

conditions (discussed in Della-Porta and Westaway, 1978). Furthermore, the implied requirement that coordinated events across the virus particle during virus entry may be inhibited by a single binding event is difficult to envision in light of the diversity of virus structures and methods for penetrating host cells.

A "multiple-hit" requirement suggests that neutralization occurs when an individual virion is bound by antibody with a stoichiometry that exceeds a required threshold, and is reversible (Burnet et al., 1937) (reviewed by Parren and Burton, 2001). Neutralization can therefore be considered from a reductionist perspective focused on the number of antibodies bound to the virion during critical steps of the virus entry process. A review of data supporting a multiple-hit mechanism of neutralization of flaviviruses is provided by Della-Porta and Westaway (1978). Evidence consistent with this perspective discussed within includes: i.) the epitope occupancy requirements for neutralization of flaviviruses, ii.) an estimate of the stoichiometric requirements for neutralization, iii.) the existence of a fraction of viruses resistant to neutralization at any concentration of antibody, iv.) the phenomenon of antibody-dependent enhancement of infection, and v.) the augmented neutralizing activity of anti-flavivirus antibodies in the presence of complement.

## Antibody affinity controls the percentage of viral epitopes bound by antibody

At least two factors define conditions that allow engagement of virions by antibody with a stoichiometry that exceeds the threshold requirements for neutralization. One of these factors, antibody affinity, defines the fraction of epitopes bound by antibody molecules at any particular concentration (Klasse and Sattentau, 2002). For example, incubation of a virion that displays 180 epitopes with antibody at a concentration equal to its $K_d$ will result in occupancy of virus particles with an average of 90 antibody molecules (Fig. 2A). Thus, it is not surprising that in many instances, antibody affinity correlates with

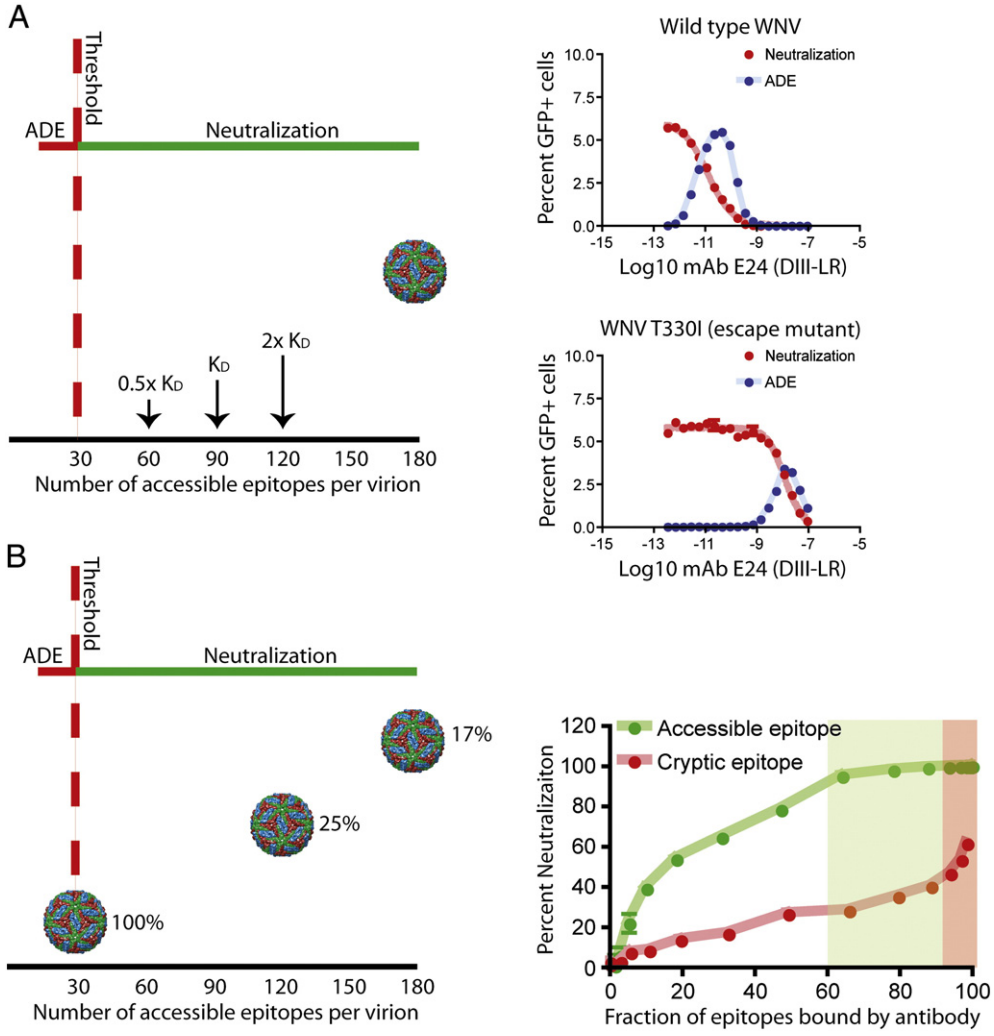


**Fig. 2.** Antibody affinity and epitope accessibility govern the neutralization potency of anti-flavivirus antibodies. Neutralization of flavivirus virions requires engagement of the virion by antibody with a stoichiometry that exceeds a threshold (estimated as 30 mAbs based on studies with two WNV E-DIII-LR-specific mAbs) (Pierson et al., 2007). Engagement of the virion with a stoichiometry below this threshold may support antibody-dependent enhancement of infection (ADE). A) Antibody affinity determines the percentage of epitopes bound by antibody at any non-saturating concentration. For example, incubation of a theoretical virus displaying 180 E protein epitopes with antibody at a concentration equal to its $K_d$ results in engagement of the virion with a stoichiometry of an average of 90 antibody molecules. Reducing the concentration two-fold (0.5× of the antibody $K_d$) translates into engagement by 60 antibodies, as determined by the relationship $(\text{Bound}/\text{Bound}_{max}) = ([Ab]/([Ab] + K_d))$. (Right panel) Mutations that change the affinity of antibody–virion interactions, such as the single T330I substitution introduced into the WNV E protein. B) Epitope accessibility governs the occupancy requirements for neutralization. For a theoretical flavivirus displaying 180 epitopes, an epitope occupancy of 17% is required to exceed a stoichiometric threshold of 30 antibody molecules. A reduction in epitope accessibility translates into increases in the fraction of epitopes that must be engaged in order to support virus neutralization. (Right panel) Epitope accessibility varies considerably for antibodies that bind structurally distinct epitopes on the flavivirus. Antibodies that bind highly accessible determinants my completely neutralize infection at relatively modest occupancy (60%, green shading), whereas antibodies that bind poorly accessible structures neutralize infection only at full occupancy (100%, red shading).
Figures and data were modified from (Pierson et al., 2008; Pierson et al., 2007).

neutralizing activity in vitro. Under non-saturating conditions, antibodies that bind viral antigens via high affinity interactions will dock onto the virion in greater numbers than those that bind with low-affinity to the same epitope. In this regard, vaccination strategies that elicit high-affinity antibodies are desirable, as this translates into increased neutralization at lower concentrations of antibody. Conversely, antigenic variants that reduce the strength of antibody binding are less sensitive to neutralization as a function of reduced occupancy at any non-saturating concentration of antibody. For example, the recent demonstration that genotypic variation of DENV reduces the neutralizing activity of antibodies against heterologous strains of the same DENV serotype can be explained by differences in antibody affinity (Brien et al., 2010; Sukupolvi-Petty et al., 2010; Wahala et al., 2010).

## Epitope accessibility is a critical and dynamic factor that governs antibody-mediated neutralization of viruses

Not all antibodies that bind virions with high affinity are characterized by significant neutralizing activity. For example, monoclonal antibodies (mAbs) E16 and E53, described in detail below, bind structurally distinct epitopes on the West Nile virus (WNV) E protein with high affinity, yet differ in their capacity to neutralize infection by several orders of magnitude (Nelson et al., 2008; Oliphant et al., 2005; Oliphant et al., 2006; Pierson et al., 2007). Antibody affinity determines the fractional occupancy of epitopes on the surface of the virion, not the number of antibody molecules bound to the virion under any condition. Thus, insight into the affinity of antibody–virion interactions is not sufficient to predict whether a particular antibody can exceed the stoichiometric requirements for neutralization. The stoichiometry of antibody binding is determined both by antibody affinity and by the total number of epitopes on the virion that are accessible for binding. Epitope accessibility defines the occupancy requirements for neutralization (Fig. 2B), which can vary considerably for antibodies that bind flaviviruses. For example, the occupancy requirements for neutralization by mAbs specific for a type-specific epitope on E-DIII of DENV serotype 2 (DENV-2) were markedly lower than the fractional occupancy required for neutralization via adjacent, partially overlapping, group- and sub-complex-reactive epitopes (Gromowski and Barrett, 2007; Gromowski et al., 2008; Gromowski et al., 2010). In agreement, the occupancy requirements for a panel of WNV-reactive mAbs were determined and varied considerably as a function of the predicted accessibility of the epitope on the mature virus particle (Pierson et al., 2007). The widely varying occupancy requirements for neutralization of flaviviruses directly support the multiple-hit model. Factors that govern epitope accessibility and the occupancy requirements of neutralization are discussed in the succeeding sections.

### *Steric constraints arising from virion structure*

The dense herringbone arrangement of E proteins on the mature flavivirus imposes steric constraints on antibody recognition. In fact, none of the mAbs characterized to date using structural methods are capable of binding all 180 E proteins incorporated into the virion (Cherrier et al., 2009; Kaufmann et al., 2006; Kaufmann et al., 2010; Lok et al., 2008; Nybakken et al., 2005). For example, the neutralizing mAb E16 binds a discontinuous epitope on the upper lateral ridge of domain III of the E protein (DIII-LR) (Nybakken et al., 2005; Oliphant et al., 2005). Cryoelectron microscopic (cryo-EM) reconstructions of Fab fragments of E16 bound to WNV demonstrate that E16 binds a maximum of 120 epitopes on the mature virion (Kaufmann et al., 2006). E16 cannot bind E proteins proximal to the five-fold symmetry axis due to steric constraints imposed by the very close proximity of neighboring DIII molecules (Fig. 1C). Antibody occupancy of a relatively small fraction (~25%) of the 120 accessible DIII-LR epitopes is required for neutralization (Fig. 2B) (Pierson et al., 2007).

In contrast, many WNV- and DENV-reactive antibodies neutralize infection only at maximal occupancy, in the presence of saturating concentrations of antibody. Residues that contribute significantly to antibody binding have been mapped for large panels of flavivirus mAbs (Beltramello et al., 2010; Brien et al., 2010; Gromowski and Barrett, 2007; Gromowski et al., 2008, 2010; Kiermayr et al., 2009; Mandl et al., 1989; Oliphant et al., 2005, 2006; Shrestha et al., 2010; Sukupolvi-Petty et al., 2010, 2007). In many cases, plotting the location of these residues on the reconstruction of the intact mature virus particle does not provide a clear structural basis for antibody recognition due to accessibility considerations. For example, the highly conserved fusion loop at the top of E domain II (DII-FL) recognized by mAb E53 is buried between DI and DIII of the opposing E protein of the dimers arranged on the mature virion (Cherrier et al., 2009; Mukhopadhyay et al., 2005). Antibodies specific for these poorly accessible determinants have to bind essentially all of them in order to exceed the stoichiometric threshold of neutralization (Nelson et al., 2008; Stiasny et al., 2006). Importantly, many antibodies that can bind virions display very little in vitro neutralizing activity because exceeding this threshold is not possible at any concentration of antibody (Nelson et al., 2008; Oliphant et al., 2006; Pierson et al., 2007). The limited neutralizing activity of antibodies specific for the prM protein may also reflect an inability to exceed the threshold requirements for neutralization.

### *The impact of structural dynamics*

The utility of molecular models of viral proteins and their organization on virions are powerful tools that reveal the average state of proteins on the virion. Estimates of epitope accessibility based on these structural models may be complicated by the possibility that virions are in constant motion. It is well established that proteins sample many conformations at equilibrium (reviewed by Boehr and Wright, 2008). This is also undoubtedly true of proteins incorporated into virus particles (reviewed in Johnson, 2003; Witz and Brown, 2001). For example, limited proteolysis studies of picornaviruses suggest the capsids of these viruses "breathe" (Bothner et al., 1998). Interestingly, a small molecule inhibitor that blocks capsid dynamics neutralizes infectivity (Lewis et al., 1998). Poliovirus VP4 is recognized by neutralizing antibodies even though this protein is not predicted to be accessible on the exterior of the particle (Li et al., 1994), implying that epitopes transiently and reversibly exposed on virions via structural dynamics may be stabilized by antibody recognition.

Viral dynamics may also have a role in the accessibility of epitopes on flaviviruses. The mAb 1A1D-2 is a DENV sub-complex specific mAb that recognizes an epitope on the A-strand of E-DIII and is capable of neutralizing DENV serotypes 1, 2, and 3 (Lok et al., 2008; Roehrig et al., 1998). Analysis of the accessibility of residues involved in 1A1D-2 recognition identified steric conflicts that should prevent binding to DIII in all three symmetry environments of the mature virus particle, consistent with an inability to dock Fab fragments of this antibody onto virions at 4 °C (Lok et al., 2008). Interestingly, antibody binding was possible when incubations were carried out at 37 °C. Cryo-EM reconstructions of 1A1D-2 in complex with DENV revealed a marked rotation of the E proteins away from the surface of the virion as compared to the arrangement of E proteins in the average state of the mature virion. The structure of 1A1D-2 bound to DENV provides insight into one of many potential conformations sampled by DENV at equilibrium, and demonstrates how such structural changes can modulate epitope accessibility (Fig. 3). Whether viral dynamics play a more widespread role in neutralization via other epitopes remains to be determined.

### *Changes in oligomeric state occurring during virion maturation*

Populations of flaviviruses released from infected cells contain not only immature and mature virions, but also virus particles that share

structural features of both these forms. While cleavage of prM is required for the production of an infectious particle (Elshuber et al., 2003), it may be inefficient. In fact, recent studies suggest that as many as 92% of DENV virions incorporate prM proteins that are not cleaved during virus egress (Junjhon et al., 2010). At least some of these partially mature virions remain infectious, although whether there is a limit to the number of uncleaved prM molecules that an infectious virion may retain is unknown (Davis et al., 2006; Guirakhoo et al., 1992). While the structure of partially mature virions is not yet known, the E proteins on these heterogeneous virus particles likely exist as both anti-parallel dimers that lie flat against the viral membrane (characteristic of mature virions), as well as spikes that project off the surface of the virion (characteristic of immature virions). E proteins in these distinct conformations and oligomeric states present very different surfaces for antibody recognition (Guirakhoo et al., 1992; Heinz et al., 1994).

Analysis of the neutralization profiles of several mAbs specific for epitopes predicted to be poorly accessible on the mature virion revealed a subset of virions resistant to neutralization by saturating concentrations of antibody (Nelson et al., 2008; Pierson et al., 2007). A similar pattern was observed when the number of epitopes on the virion recognized by the potently neutralizing mAb E16 was reduced using genetic methods, as discussed below (Mehlhop et al., 2009; Pierson et al., 2007). Increasing the efficiency of virus maturation yielded a homogenous population of mature WNV resistant to neutralization by mAb E53 and other mAbs specific for cryptic determinants (Nelson et al., 2008). Conversely, inhibiting maturation increased sensitivity to neutralization by these mAbs, and significantly reduced the size of the resistant fraction described above. These results suggest that the process of virion maturation modulates the sensitivity of WNV to neutralization by mAbs that recognize several distinct classes of epitopes through changes in epitope accessibility (Fig. 3C). The structural basis of this phenomenon has been confirmed. The mAb E53 binds the highly conserved fusion loop at the terminus of E-DII (DII-FL) and neutralizes WNV in a maturation state-dependent fashion. Cryo-EM reconstruction studies of Fab fragments of E53 bound to WNV and DENV demonstrated binding to E proteins only when complexed with prM in the heterotrimeric spikes of immature virions (Cherrier et al., 2009). In this context, binding to two of three fusion loop epitopes was possible; binding to the third E protein was prevented by steric conflicts between the DII-FL and prM. Due to the inaccessibility of the DII-FL epitope on the mature form of the virion (discussed above), significant binding or neutralization of E53 to mature virions was not detected.

### *Limits on epitope accessibility imposed by the steric bulk of intact antibody molecules*

Antibodies are relatively large molecules that occupy considerably more space than simply the surface area of the virion in contact with the paratope. This steric bulk is thought to contribute to neutralization by sterically preventing required interactions with cellular factors during the virus entry process or by interfering with conformational changes in viral proteins required for fusion (Klasse and Sattentau, 2002). In contrast, the size of antibody molecules may in some instances be detrimental. For example, the broadly neutralizing mAb 4E10 binds the conserved membrane proximal external region (MPER) of HIV-1 gp41. Fabs and a smaller engineered fragment of mAb 4E10 displayed greater neutralizing activity than the intact antibody molecule, presumably due to a reduction in the degree of steric occlusion (Klein et al., 2009). Antibody size may also influence the maximal number of antibody molecules that can simultaneously bind to virions (Kiermayr et al., 2009).

## Estimates of the stoichiometry of antibody-mediated neutralization of West Nile virus

The number of antibodies required for the neutralization of several virus types has been investigated (reviewed in Burton et al., 2001). The stoichiometric requirement for neutralization varies considerably among viruses. For example, neutralization of poliovirus occurs following engagement by 4–5 mAbs, whereas inhibition of rabies virus requires more than 200 IgG molecules (Flamand et al., 1993; Icenogle et al., 1983). One unifying principle of antibody-mediated neutralization is that the number of antibodies required to neutralize infection is determined by the surface area of the virus particle (Burton et al., 2001); increased virion size requires a larger number of antibody molecules to sterically interfere with required steps in the virus entry process. The stoichiometric requirement for neutralization of WNV has been studied using two antibodies specific for the DIII-LR and was estimated as ~30 antibody molecules (Mehlhop et al., 2009; Pierson et al., 2007). Estimates for the number of antibodies required to neutralize WNV are in agreement with the predictions of this "coating theory" (Klasse and Burton, 2007). However, the functional significance of this number is not presently clear, as the number of antibodies required for neutralization does not in itself provide insight into the mechanism of neutralization. Antibodies have the potential to block multiple steps in the virus entry process. Antibodies that block flavivirus attachment (Crill and Roehrig, 2001; He et al., 1995; Nybakken et al., 2005) and fusion (Gollins and Porterfield, 1986; Roehrig et al., 1998; Stiasny et al., 2007; Thompson et al., 2009; Vogt et al., 2009) have been described, although it is quite possible that antibodies have the potential to neutralize via multiple mechanisms depending on epitope occupancy. For example, mAb E16 blocks viral fusion by preventing the conformational changes in the E protein required to drive fusion (Kaufmann et al., 2009; Thompson et al., 2009) and has a modest effect on virus attachment at high concentrations. These observations suggest that the threshold requirements for neutralization may differ depending on the step in the virus entry/fusion pathway blocked by the antibody. Likewise, it remains to be determined whether the threshold requirements for neutralization will differ for antibodies specific for structurally distinct epitopes, as suggested by studies of mAbs that neutralize bovine papillomavirus (Booy et al., 1998; Roden et al., 1994). To date, the analysis of the stoichiometric requirements for antibodies that bind outside the DIII-LR has been complicated by the significant impact of virion maturation and/or structural dynamics on epitope accessibility.

## Antibody-dependent enhancement of infection

Antibody-dependent enhancement of infection (ADE) describes a marked increase in the efficiency of virus infection in the presence of non-neutralizing concentrations of virus-reactive antibody (Halstead and O'Rourke, 1977; Hawkes, 1964). ADE is most commonly observed using cells expressing Fc- or complement-receptors, and has been observed in vitro for several viruses (reviewed in Porterfield, 1986). The most clearly established role for ADE in vivo exists for DENV (reviewed in Halstead, 2003). Infection by any of the four serotypes of DENV results in a febrile illness called dengue fever from which that individual will typically recover and thereafter be immune to re-infection by the same serotype of DENV. However, re-infection of that same individual with a heterologous serotype of DENV may result in a more severe and potentially fatal clinical course (reviewed in Halstead, 2002). While multiple host and viral factors likely contribute to circumstances that lead to severe manifestations of disease (Green and Rothman, 2006; Halstead, 2003), one possibility is that antibodies raised during primary infection not only fail to protect from secondary infection by a heterologous DENV serotype, but also have the potential to enhance virus infection in vivo. The increased

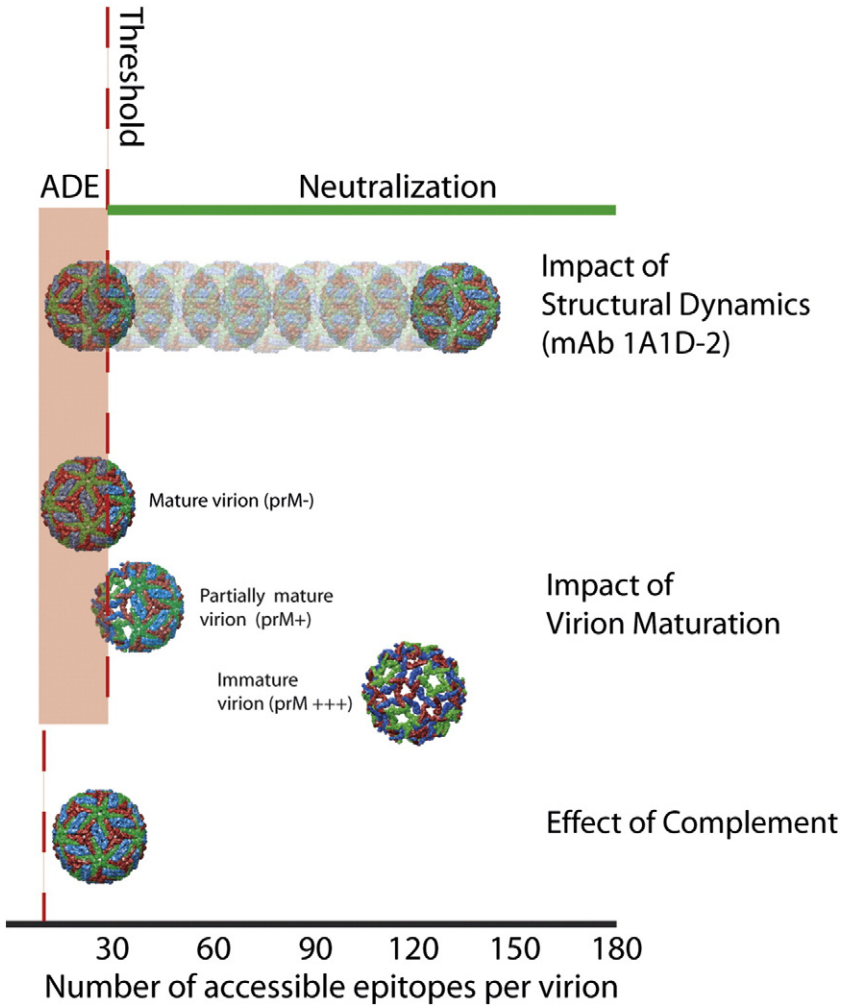


**Fig. 3.** Factors that govern the neutralizing activity of anti-flavivirus antibodies. Neutralization of flavivirus infection is a multiple hit phenomenon that requires engagement of the virion by antibody with a stoichiometry that exceeds a threshold. Whether an individual antibody can dock on the virion with a stoichiometry sufficient to exceed this threshold depends on its affinity for viral antigens and the total number of accessible epitopes displayed on the average virion (shown schematically on the x-axis). Changes in epitope accessibility significantly impact the potency of neutralizing antibodies. The dynamic motion of DENV appears to regulate exposure of the A-strand epitope recognized by mAb 1A1D-2 at equilibrium, and explains the temperature-dependence of binding by this antibody. The process of virion maturation also impacts accessibility of epitopes bound by neutralizing antibodies. Virion maturation reduces accessibility of several classes of antibodies to levels that no longer support neutralization. Interactions with the complement protein C1q increase the potency of neutralizing antibodies, even those specific for poorly accessible determinants. C1q augments neutralization by decreasing the stoichiometric threshold required for neutralization (shifted red dashed line, bottom).
Figure modified from (Pierson et al., 2008).

risk of severe disease following primary infection of children of DENV-immune mothers provides direct support for a role for antibody in DENV pathogenesis (Kliks et al., 1988, 1989). In addition, enhanced viremia has been observed following passive transfer of antibody in primates (Goncalvez et al., 2007; Halstead, 1979), and two recent reports describe the characterization of a murine model that recapitulates some aspects of severe DENV, including the role of antibody in augmenting infection in vivo (Balsitis et al., 2010; Zellweger et al., 2010).

Virus neutralization and ADE are two phenomena related by the number of antibodies bound to the virion (Morens et al., 1987; Pierson et al., 2007). ADE occurs at concentrations of antibody bounded at an upper limit by the stoichiometric threshold for neutralization, and at lower concentrations by the minimal number of antibodies required to support stable attachment of the virion to cells. Analysis of the stoichiometric requirements for ADE of K562 cells that express the Fcγ-receptor CD32 suggests that ~15 antibodies are required for enhanced infection of WNV (Pierson et al., 2007). This provides a useful perspective in which to evaluate the likelihood that antibodies of known specificity will enhance infection. Potently neutralizing antibodies support ADE when diluted past concentrations sufficient to neutralize infection. For antibodies that recognize poorly accessible determinants on the virion, which typically require full occupancy to exceed the stoichiometric threshold for neutralization, even small changes in the number of antibodies bound to the virion result in ADE. Furthermore, antibodies that recognize infrequently displayed epitopes that do not support neutralization may enhance infection even at saturation. Indeed, the enhancing properties of poorly neutralizing prM-reactive antibodies can be observed over a wide range of antibody concentrations (Beltramello et al., 2010; Dejnirattisai et al., 2010).

The cell biology of ADE remains incompletely understood. ADE is most readily observed on cell types that do not efficiently bind virus particles. Antibodies complexed to virions may significantly increase the efficiency of virus attachment to cells through multivalent interactions with Fc-receptors. ADE can be inhibited by antibodies that block Fc-receptor engagement (Peiris et al., 1981), removal of the antibody heavy chain (Zellweger et al., 2010), or deletion of the N-linked sugar on IgG molecules required for interactions with Fcγ-receptors (Balsitis et al., 2010; Goncalvez et al., 2007; Pierson et al., 2007). The expression of cellular attachment factors on Fcγ-receptor expressing cells markedly reduces the extent to which virus

infection may be enhanced in the presence of antibody (Boonnak et al., 2008; Pierson et al., 2007). For example, immature dendritic cells (DCs) do not appear to support ADE of DENV despite expressing readily detectable Fcγ-receptors presumably because they express the DENV attachment factor CD209 (DC-SIGN). In contrast, mature DCs express reduced levels of CD209 and support ADE through FcγRIIa (Boonnak et al., 2008). In some instances, ADE may occur when antibody molecules cross-react with both viral and cellular antigens. DENV prM-specific antibodies were identified that were capable of binding simultaneously to the virion and target cell, providing a Fcγ-receptor-independent mechanism for internalization and infection (Huang et al., 2006). A recent study demonstrated that 75% of mAbs cloned from HIV-1-infected patients demonstrated polyreactive specificities (Mouquet et al., 2010), however, whether flavivirus-specific antibodies with these properties are common remains unclear.

While Fc-receptor-mediated uptake of DENV immune complexes results in increased infection simply by increasing the number of susceptible cells, recent studies have suggested signaling events that occur following the ligation of Fc-receptors may be important for ADE (Moi et al., 2010; Rodrigo et al., 2006), resulting in the inhibition of the antiviral response and increased viral replication (intrinsic ADE). Specifically, intrinsic ADE is thought to occur by two separate mechanisms: 1) disruption of the RIG-I/MDA5 signaling cascade, which in turn abrogates the type I IFN response, and 2) induction of anti-inflammatory cytokines such as IL-10 coupled with suppression of pro-inflammatory cytokines such as IL-12 and TNF-α (Boonnak et al., 2008; Chareonsirisuthigul et al., 2007; Ubol et al., 2010).

## Serum complement proteins increase the neutralizing activity of antibodies via multiple mechanisms

While the engagement of virions by antibody has the potential to directly neutralize infectivity, in the host these interactions occur in the presence of serum proteins capable of modulating antibody function. The complement system is a component of the innate immune response capable of pathogen recognition and clearance that is regulated by a complex cascade of cleavage events. The importance of the complement system in viral immunity is highlighted by the number and diversity of strategies evolved by viruses to antagonize it (reviewed by Lambris et al., 2008). Activation of the complement system occurs via three convergent pathways that ultimately lead to the release of anaphylatoxins and chemotactic factors, deposition of the opsonic complement protein C3b on the surface of the pathogen, and the assembly of the membrane attack complex (MAC) capable of pathogen lysis. The classical pathway of complement activation is triggered by interactions between the complement protein C1q and the Fc-portion of antibody molecules. C1q is a ~460 kDa multimeric protein composed of six globular heads connected at the amino-terminus by collagenous flexible stalks (reviewed by Kishore and Reid, 2000). While C1q can bind efficiently to a single pentameric IgM molecule bound to the surface of a pathogen, the affinity of monomeric interactions with IgG is quite low. Efficient interactions with IgG molecules require multivalent interactions with antibody molecules arrayed on the surface of the pathogen, and occur in an isotype-dependent manner. In humans, IgG3 and IgG1 efficiently interact with C1q (IgG3>IgG1), whereas C1q binding to IgG2 and IgG4 subclasses is very weak (the hierarchy of C1q binding to murine antibodies is IgG2a>IgG2b>IgG1) (Leatherbarrow and Dwek, 1984; Schumaker et al., 1976).

An understanding of the impact of complement on the potency of antibodies has advanced considerably since the first reports that heat-labile factors in serum augment the neutralizing activity of antibodies. Complement has been shown to increase the neutralization activity of antibodies against many viruses, including flaviviruses (Della-Porta and Westaway, 1978). Several lines of evidence suggest that C1q is sufficient for enhancing the neutralizing activity of antibodies that bind flaviviruses (Mehlhop et al., 2009). Incubation of virion–antibody complexes with fresh, but not heat-inactivated sera, results in more efficient neutralization in vitro; this effect is lost when serum obtained from C1q- but not C3- or C5-deficient mice is added to neutralization experiments. The addition of recombinant C1q to neutralization tests also results in increased neutralization in an antibody isotype-dependent fashion. Together, these data suggest that molecules in the complement cascade downstream of C1q activation, including the formation of the MAC on virions, are not required for enhanced neutralization of flaviviruses. Studies of the mechanism of complement-enhanced neutralization of influenza virus reached similar conclusions (Feng et al., 2002). Neutralization of the extracellular enveloped form of vaccinia virus (EV) by B5R-specific antibodies was antibody isotype- and C1q-dependent, but also required deposition of C3 (but not C5 activity) (Benhnia et al., 2009).

C1q has the potential to directly increase the neutralizing activity of antibodies through one or more mechanisms. Because C1q only binds efficiently to multiple antibody molecules arrayed on the surface of the virus particle (Kishore and Reid, 2000), C1q engagement may increase the functional avidity of antibodies for the virion. This would translate into enhanced neutralization via increased antibody occupancy at any non-saturating concentration of antibody. Multivalent C1q interactions also cross-link antibodies (and viral proteins) on the surface of the virus particle together, which may have significant implications for the viral fusion machinery (Kaufmann et al., 2009; Whiteman et al., 2010). Finally, the relatively large size of C1q adds substantially to the bulk of antibody molecules bound to the virion, which may increase neutralizing activity due to enhanced steric effects.

Our studies with WNV suggest that C1q enhances antibody-mediated neutralization by significantly reducing the stoichiometric requirements for neutralization (Mehlhop et al., 2009). Neutralization of WNV can be controlled by manipulating the number of epitopes displayed on the average virion using genetic approaches (Pierson et al., 2007) or via changes in the maturation state of the virus particle (Nelson et al., 2008), as discussed above. Reducing the number of epitopes on the virion below the stoichiometric threshold required for neutralization results in the appearance of a fraction of viruses resistant to neutralization, even when incubated with saturating concentrations of antibody. The addition of C1q to neutralization studies dramatically reduced the size of this resistant fraction in an isotype-dependent manner. In this context, C1q-mediated increases in avidity are not sufficient to explain this increase in neutralization because all of the accessible epitopes on the virion are already bound by saturating concentrations of antibody. Instead these results suggest that C1q increases neutralizing activity through significant changes in the stoichiometric threshold for neutralization (Fig. 3). This effect could be mimicked by the incubation of virion–antibody complexes with Fab2 molecules specific for IgG, but not Fab fragments of the same specificity incapable of bivalent interactions with antibodies bound to the virion. These findings rule out a requirement for the very large size of the C1q molecule, and suggest that cross-linking is mechanistically important. A reduced stoichiometric requirement for neutralization in the presence of C1q also provides a mechanism to explain complement modulation of ADE (Mehlhop et al., 2007; Yamanaka et al., 2008).

## Toward understanding the complexities of the polyclonal antibody response

Experiments with mAbs have yielded an increasingly sophisticated understanding of the molecular mechanisms of flavivirus neutralization. A quantitative view of neutralization provides a framework to define the functional and biochemical features of the most potently

neutralizing antibodies, as well as those with the greatest potential to enhance infection. The identification of antibodies with therapeutic value is an exciting practical product of these efforts (Morrey et al., 2006). However, relatively little is known about the composition and functional properties of antibodies elicited by flavivirus infection. The antibody response to infection is likely a polyclonal ensemble of antibodies specific for structurally distinct epitopes on the virus particle. While many of the most potent neutralizing antibodies bind epitopes in E-DIII (Beasley and Barrett, 2002; Gromowski and Barrett, 2007; Oliphant et al., 2005), recent studies of human mAbs isolated from immortalized B cells or the analysis of combinatorial libraries of B cell donors suggest the repertoire of antibodies produced in vivo is focused elsewhere (Beltramello et al., 2010; Dejnirattisai et al., 2010; Throsby et al., 2006). Cross-reactive E-DII-reactive antibodies characterized by limited neutralization activity are common. This conclusion is supported by biochemical and serologic analysis of antibody reactivity (Crill et al., 2009, 2007; Lai et al., 2008; Oliphant et al., 2007). While it is possible that low concentrations of potently neutralizing E-DIII-reactive antibodies could be functionally significant (Crill et al., 2009), two approaches suggest this is not the case. Ablation of the DIII-LR epitope on WNV had little to no effect on the neutralization titers of antibody in the sera of infected humans or horses (Nelson et al., 2008; Oliphant et al., 2007; Sanchez et al., 2007). In agreement, a recent study demonstrated that the depletion of the DIII-reactive antibodies present in DENV-immune sera did not have a significant impact on neutralization when compared to non-depleted sera (Wahala et al., 2009). While the analysis of the functional interplay between components of the polyclonal antibody response is at a relatively early stage, it holds great promise for the identification of epitopes associated with protective responses, insight into circumstances that may contribute to augmented virus infection in vivo, and more focused targets for immunization.

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