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Short Article

Complement Protein C1q Inhibits Antibody-Dependent Enhancement of Flavivirus Infection in an IgG Subclass-Specific Manner

Erin Mehlhop,[1] Camilo Ansarah-Sobrinho,[4] Syd Johnson,[5] Michael Engle,[2] Daved H. Fremont,[1] Theodore C. Pierson,[4] and Michael S. Diamond[1,2,3,*]
[1]Department of Pathology and Immunology
[2]Department of Medicine
[3]Department of Molecular Microbiology
Washington University School of Medicine, St. Louis, MO 63110, USA
[4]Viral Pathogenesis Section, Laboratory of Viral Diseases, National Institutes of Health, Bethesda, MD 20892, USA
[5]MacroGenics, Inc., Rockville, MD 20850, USA
*Correspondence: diamond@borcim.wustl.edu
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SUMMARY

Severe dengue virus infection can occur in humans with pre-existing antibodies against the virus. This observation led to the hypothesis that a subneutralizing antibody level in vivo can increase viral burden and cause more severe disease. Indeed, antibody-dependent enhancement of infection (ADE) in vitro has been described for multiple viruses, including the flaviviruses dengue virus and West Nile virus. Here, we demonstrate that the complement component C1q restricts ADE by anti-flavivirus IgG antibodies in an IgG subclass-specific manner in cell culture and in mice. IgG subclasses that avidly bind C1q induced minimal ADE in the presence of C1q. These findings add a layer of complexity for the analysis of humoral immunity and flavivirus infection.

INTRODUCTION

The genus *Flavivirus* is composed of 73 viruses, ~40 of which are associated with severe human diseases including West Nile (WNV), yellow fever (YFV), Japanese encephalitis (JEV), and dengue (DENV) viruses (Burke and Monath, 2001). This clinically important group of arthropod-borne viruses shares a common nucleic acid genome structure (positive-polarity, ~11 kilobase single-stranded RNA), organization (three structural and seven nonstructural proteins), particle size (~500 Å), and cellular life cycle. Studies by several groups have established that humoral immunity to flavivirus infection is necessary and sufficient for host protection from disease (Ben-Nathan et al., 2003; Diamond et al., 2003a, 2003b; Oliphant et al., 2005, 2006; Roehrig et al., 2001; Schlesinger et al., 1985; Tesh et al., 2002). Following infection, the majority of neutralizing antibodies are directed against the flavivirus envelope (E) protein, although some likely recognize the premembrane/membrane (prM/M) protein (Colombage et al., 1998; Falconar, 1999; Pincus et al., 1992; Vazquez et al., 2002). Antibody protection in vivo generally correlates with neutralizing activity in vitro (Kaufman et al., 1987; Phillpotts et al., 1987; Roehrig et al., 2001). However, Fc-dependent effector functions also contribute to the protective activity of at least some anti-flavivirus antibodies in vivo (Oliphant et al., 2005).

Paradoxically, Fc-γ receptor (Fc-γR) engagement by antibodies in vitro also has been observed to enhance replication of flaviviruses (Gollins and Porterfield, 1984, 1985; Halstead and O'Rourke, 1977; Kliks, 1990; Kliks et al., 1989; Peiris et al., 1981; Peiris and Porterfield, 1979). At concentrations that do not reach the stoichiometric threshold necessary for neutralization, anti-flavivirus antibodies enhance infection in cells expressing activating Fc-γR (Pierson et al., 2007). This phenomenon, also known as antibody-dependent enhancement of infection (ADE) is hypothesized to contribute to the pathogenesis of secondary DENV infection (Halstead, 2003), and possibly, the adverse effects following challenge of individuals immunized with some formalin-inactivated viral vaccines (Iankov et al., 2006; Ponnuraj et al., 2003; Porter et al., 1972; Prabhakar and Nathanson, 1981). Despite its extensive characterization in vitro, and its possible epidemiological link to the pathogenesis of dengue hemorrhagic fever (DHF), the significance of ADE for DENV and other flaviviruses in vivo remains controversial (Barrett and Gould, 1986; Goncalvez et al., 2007; Gould and Buckley, 1989; Gould et al., 1987; Halstead, 1979; Rosen, 1989; Wallace et al., 2003). Part of this controversy stems from an inability to establish reproducible models of ADE in small animal models.

The Fc region of IgG also activates the complement system through the classical pathway (Volanakis, 2002). Complement is a family of serum proteins that interact in a serine protease catalytic cascade leading to the release of proinflammatory peptides, attachment of opsonins, and formation of the membrane attack complex (MAC). The

complement opsonin C1q binds to the heavy chain CH_2 constant region of IgG (Duncan and Winter, 1988; Idusogie et al., 2000) and activates the classical pathway C3 convertase, which promotes C3b opsonization and formation of the C5-C9 MAC (Volanakis, 2002). Complement activation augments the neutralizing activity of antiviral antibodies against measles (Iankov et al., 2006), influenza (Feng et al., 2002; Mozdzanowska et al., 2006), vesicular stomatitis (Beebe and Cooper, 1981), hepatitis C (Meyer et al., 2002), and human immunodeficiency (Aasa-Chapman et al., 2005; Spruth et al., 1999) viruses. In contrast, the addition of serum complement to anti-WNV IgM enhanced infection in macrophages (Cardosa et al., 1983, 1986).

Herein, we investigate the role of complement in modulating ADE of anti-flavivirus IgG. We identify C1q as the serum component necessary and sufficient to restrict ADE in vitro in an IgG subclass specific manner. Based on these findings, we used $C1q^{-/-}$ mice to demonstrate in vivo the IgG subclass-specific requirements for the development of ADE.

RESULTS

At subneutralizing concentrations, antibody can enhance infection of flaviviruses in Fc-γR-expressing cells (Halstead, 2003; Pierson et al., 2007). To test for the effect of C1q or any specific complement component on ADE, we used a highly quantitative, flow cytometric-based functional assay with WNV reporter virus particles (RVP) (Pierson et al., 2006, 2007). RVP are virus-like particles composed of the structural proteins of WNV and a subgenomic replicon encoding a reporter gene. RVP are capable of only a single round of infection and allow virus entry to be measured as a function of reporter gene activity. WNV RVP were incubated with purified mouse mAbs in the presence of fresh mouse serum prior to infection of K562 cells, a human erythroleukemia cell line that expresses high levels of the activating Fc-γ receptor IIa (FcγRIIa) and has been used to study ADE of flaviviruses in vitro (Littaua et al., 1990; Pierson et al., 2007). In the absence of serum, RVP mixed with serial dilutions of a strongly neutralizing IgG_{2b} mAb (E16) that binds domain III (DIII) of WNV E protein (Oliphant et al., 2005) promoted a biphasic infection pattern characteristic of ADE (Morens et al., 1987; Pierson et al., 2006, 2007). Strikingly, the addition of 5% fresh mouse serum almost completely abolished E16-mediated enhancement, reducing the peak WNV infection by ~37-fold (Figure 1A, $p = 0.002$, $n = 9$). The serum-dependent inhibition of ADE was inactivated by heat and required C1q but not C3, mannose-binding lectins (MBL), factor B (fB), or C5 as determined by experiments with $C1q^{-/-}$, $C3^{-/-}$, $MBL^{-/-}$, $fB^{-/-}$, or $C5^{-/-}$ serum (Figure 1B and data not shown). Purified C1q elicited the same effect as wild-type mouse serum, reducing peak E16-enhancement ~60-fold ($p = 0.02$, $n = 4$). Similar results were observed with E24, an epitope-matched IgG_{2a} (Figures 1C and 1D). However, neither serum nor purified C1q inhibited ADE induced by the epitope-matched IgG_1 mAb, E34 (Figure 1E, $p > 0.8$, $n = 3$, and data not shown). Studies were repeated with three, more weakly neutralizing DII fusion-loop-specific anti-E antibodies, E18, E28, and E60, which enhance WNV infection in cells expressing Fc-γR (Oliphant et al., 2006). Analogously, purified C1q inhibited ADE induced by the IgG_{2a} mAbs E18 and E60 (43- to 136-fold, Figures 1F and 1G, $p \leq 0.04$, $n > 3$), but not that induced by the epitope-matched IgG_1 mAb E28 (Figure 1H).

Mouse macrophages express multiple activating Fc-γR (I, III, and IV) and are permissive for WNV in vivo (Samuel and Diamond, 2005). To determine the effect of C1q in a more relevant in vitro infection model, primary mouse macrophages were exposed to infectious WNV (New York 2000 strain) that was preincubated with antibody in the presence of mouse serum or purified C1q. Analogous to the RVP assays, we observed a 35-fold enhancement of infection in the presence of E16 (Figure 1K), and peak ADE was inhibited ~18-fold by the addition of either mouse serum or purified C1q ($p \leq 0.001$, $n = 6$). Correspondingly, no difference in enhancement was observed using the epitope-matched IgG_1 mAb, E34 (Figure 1L). These data confirm that C1q reduces ADE of live WNV infection in cell types that express multiple Fc-γR and are relevant in vivo.

To verify that this phenotype was not specific to mouse mAbs, we engineered human IgG subclass switch variants of E16 and tested for ADE in cells expressing human Fc-γRIIa. As expected, ADE by the four human IgG subclasses of E16 was observed (Figure 2). However, there was an ~50-fold difference in the peak ADE induced by the different switch variants, corresponding to the relative affinity of the IgG subclasses for Fc-γRIIa (Hulett and Hogarth, 1994; Ravetch and Kinet, 1991). When C1q was added, peak ADE by E16 hu-IgG_3 was markedly diminished (9-fold, $p < 0.0001$, $n = 8$, Figure 2C). In contrast, C1q had no effect on ADE by E16 hu-IgG_2 (Figure 2B) or hu-IgG_4 (Figure 2D) and a lesser inhibitory effect on E16 hu-IgG_1 (~3-fold, $p = 0.03$, $n = 5$, Figure 2A). The effect on E16 hu-IgG_1-mediated ADE by C1q was not dose-dependent, as similar fold reductions were observed when C1q concentrations were varied in the physiologic range between 50 and 200 μg/ml (Figure S1A in the Supplemental Data available with this article online). These data establish that C1q inhibits ADE of human anti-flavivirus IgG in a subclass-dependent manner.

As only a small subset of individuals with pre-existing anti-DENV antibodies develop DHF on secondary challenge with a heterologous serotype (Halstead, 1989), we hypothesized that C1q binding to antibody-opsonized DENV could restrict ADE and possibly disease. To begin to address this, DENV type 1 (DENV-1) RVP were incubated with increasing concentrations of two flavivirus crossreactive, DII-specific, epitope-matched mAbs E18 (IgG_{2a}) and E28 (IgG_1) in the presence of purified C1q prior to infection. In the absence of C1q, incubation with E18 or of E28 resulted in an average peak of ~25- and 108-fold enhancement of infection, respectively (Figures 1I and 1J). Purified C1q inhibited E18-dependent ADE ~43-fold ($p = 0.04$, $n = 4$), but not that induced by E28 ($p > 0.6$,

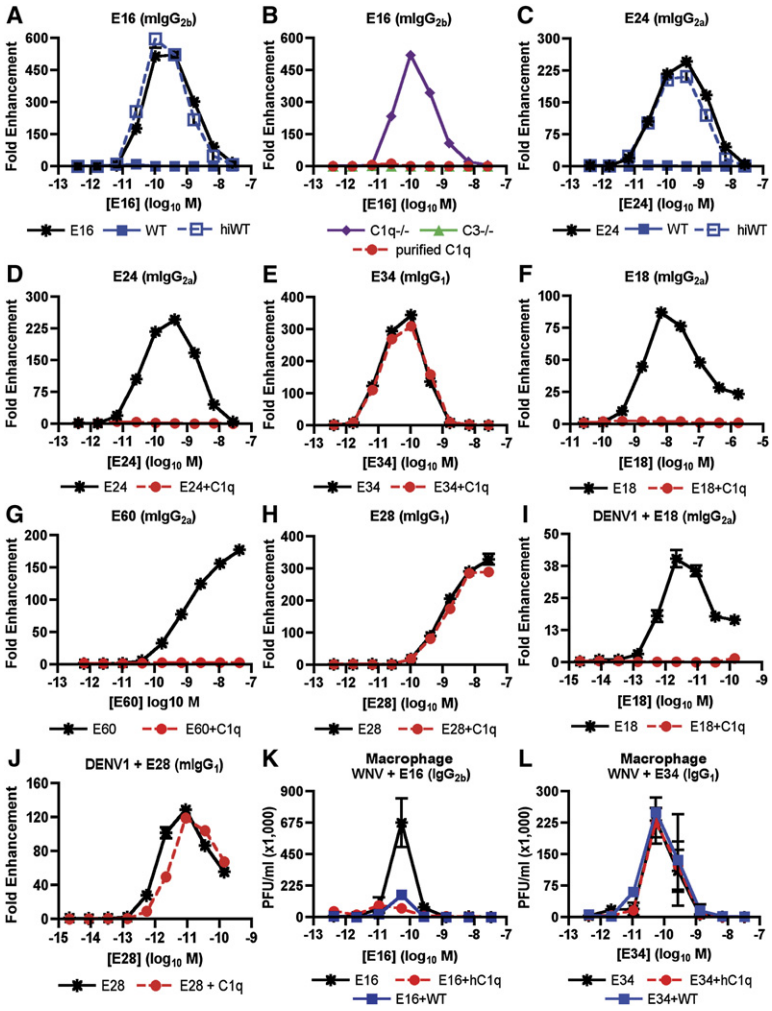

Figure 1. Mouse Serum or C1q Modulates mAb Enhancement of WNV and DENV Infection

(A) Serial dilutions of E16 (mouse IgG_{2b}) were mixed with media and 5% fresh or heat-inactivated mouse serum, incubated with WNV RVP, and added to $Fc\gamma RIIa^{+}$ K562 cells. Forty-eight hours later, cells were analyzed by flow cytometry for GFP expression. The data are expressed as the fold enhancement of infection compared to no antibody, and one representative experiment of five is shown.

(B) Experiments were performed as in (A) except that fresh $C1q^{-/-}$ or $C3^{-/-}$ serum or purified C1q (50 μg/ml) was mixed with the mouse E16 mAb.

(C–E) Experiments were performed as in (A) and (B) except that the epitope matched DIII-specific E24 (mouse IgG_{2a}) (C and D) or E34 (mouse IgG_1) (E) mAbs were used.

(F and G) Experiments were performed as above except that the flavivirus crossreactive DII-specific IgG_{2a} mAbs E18 (F) or E60 (G) were used.

(H) Experiments were performed as above except the flavivirus crossreactive DII-specific IgG_1 mAb E28 was used.

(I and J) Serial dilutions of the flavivirus crossreactive E18 (IgG_{2a}) (I) or E28 (IgG_1) (J) were mixed with DENV1 RVP in the presence or absence of C1q and added to K562 cells.

(K and L) Serial dilutions of E16 (IgG_{2b}) (K) or E34 (IgG_1) (L) were mixed with infectious WNV in the presence or absence of C1q and added to mouse macrophages. One day later supernatants were harvested and titered by plaque assay. The data from one representative experiment of six are shown and expressed as infectious plaque-forming units (PFU) per ml. Statistical analysis is described in the text. Error bars indicate standard deviations.

n = 2). Thus, C1q also limits ADE of DENV infection in an IgG subclass-specific manner.

Polyclonal antibodies against viruses are of mixed IgG subclass, antigen specificity, and affinity. To determine if C1q modulates ADE induced by polyclonal antibodies, we incubated WNV or DENV RVP with C1q and immune IgG from WNV-challenged mice, heat-inactivated serum from convalescent WNV patients, or umbilical cord blood samples from DENV-immune mothers. The latter specimen are particularly relevant, as they contain IgG that is passively transferred to the fetus during gestation; these antibodies are believed to account for the increased risk of severe DHF/Dengue shock syndrome (DSS) during the first 6–9 months of human life (Kliks et al., 1988). Mouse immune IgG against WNV enhanced infection ~350-fold compared to nonimmune murine IgG (Figure 3A, $p = 0.03$, $n = 3$), and purified human C1q reduced this enhancement ~39-fold ($p = 0.02$, $n = 3$). Similarly, all WNV and DENV patient immune sera enhanced WNV and DENV-1 infection (Figures 3B–3F and Figure S2), respectively. However, marked differences in the ability of C1q to inhibit ADE were observed. For example, in some patient sera, C1q nearly abolished ADE, reducing enhancement ~25-fold (Figure 3C, $p = 0.05$, $n = 3$, Figure S2), whereas in others the effect was modest, with an ~3- to 4-fold reduction (Figures 3B, 3D, and 3F, Figure S2, $p \leq 0.02$, $n = 3$). Finally, in other samples (Figure 3E), C1q had virtually no effect on reducing ADE. Analogous to experiments with hu-E16 mAbs, higher concentrations of purified C1q did not further restrict ADE by polyclonal sera (Figure S1B). Thus, C1q modulates WNV and DENV ADE induced by polyclonal antibodies, but to different degrees depending on the individual sample.

Based on our in vitro data, we hypothesized that C1q could limit ADE in vivo. To test this, wild-type and $C1q^{-/-}$ mice were passively administered DII (E18 and E28) and DIII-specific (E16, E24, and E34) mouse mAbs of different IgG subclasses 1 day prior to WNV infection. Four days later, spleen tissues were titrated for viral burden. The spleen was examined because Fc-γR-expressing macrophages are targets for WNV infection in this tissue (Samuel and Diamond, 2005). Notably, ADE was

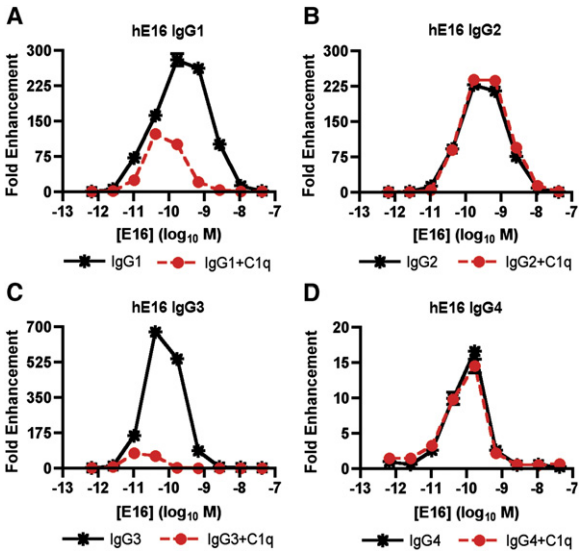

Figure 2. C1q Modulates ADE by Human IgG Subclass Switch Variants of E16 in K562 Cells

Serial dilutions of E16 hu-IgG1 (A), hu-IgG2 (B), hu-IgG3 (C), and hu-IgG4 (D) were mixed with WNV RVP in the presence or absence of C1q prior to infection of FcγRIIa$^+$ human K562 cells. Cells were harvested 48 hr after infection and processed by flow cytometry. The data are expressed as the fold enhancement of infection compared to no antibody, and one representative experiment of three is shown. Statistical analysis is described in the text. Error bars indicate standard deviations.

detected significantly at only one mAb dose (0.1 ng) and at low levels (1.5- to 2.3-fold, $p \leq 0.05$) in wild-type mice receiving the DII- or DIII-specific mIgG$_1$ mAbs, which bind C1q poorly (Figures 4A and 4B). As expected, this modest enhancement was abrogated in congenic mice that genetically lack activating Fc-γR (Figures 4D and 4E). ADE was not observed ($p > 0.7$) at any dose in wild-type mice with DII-specific E18 (mIgG$_{2a}$) or DIII-specific E16 (mIgG$_{2b}$) mAbs, which are predicted to bind C1q avidly (Figures 4A and 4B). Passive transfer experiments were also performed in wild-type mice with human IgG subclass variants of E16 that bind C1q poorly (IgG$_2$ and IgG$_4$) or strongly (IgG$_3$); no significant ADE of WNV infection in vivo was observed with any of these E16 human IgG variants (Figure 4C). In contrast, passive transfer of 10 and 100 ng of the DII-specific E18 (mIgG$_{2a}$) to *C1q*$^{-/-}$ mice enhanced WNV infection 4- to 15-fold in the spleen (Figure 4F, $p \leq 0.05$). Transfer of 100 ng of the DII-specific E28 (mIgG$_1$) also showed modest enhancement in the absence of C1q. Analogously, passive transfer of several doses of the DIII-specific E24 (mIgG$_{2a}$) strongly enhanced WNV infection (1.5- to 22-fold, $p \leq 0.05$) in *C1q*$^{-/-}$ mice, whereas transfer of the epitope-matched E34 (mIgG$_1$) had little enhancing effect (Figure 4G). Somewhat unexpectedly, E16, the DIII-specific mouse IgG$_{2b}$, did not promote significant ADE in the spleen of *C1q*$^{-/-}$ mice, despite testing across a 4-log dose range (Figure 4G). Similar results were observed with the two human IgG subclass variants of E16 that most avidly bind C1q; despite robust ADE in vitro in human K562 cells in the absence of C1q (see Figure 2), little, if any, ADE was observed in *C1q*$^{-/-}$ mice with E16 hu-IgG$_1$ or hu-IgG$_3$ over a wide range of antibody doses (Figure 4H). Thus, in wild-type mice, ADE of WNV infection occurs in vivo in a very limited manner, and only with mAbs (mIgG$_1$) that bind C1q poorly. In *C1q*$^{-/-}$ mice, ADE occurs more significantly with some mAbs of the mIgG$_{2a}$ subclass. Although we reproducibly detected ADE of WNV infection in vivo with specific mAbs, no increase in lethality or change in disease phenotype was observed (data not shown), possibly because of the relatively flat dose-response curve of WNV disease in C57BL/6 mice (Diamond et al., 2003a).

DISCUSSION

Although ADE is readily observed in vitro with a number of viruses, it has been difficult to establish consistently in vivo in animal models. While our data suggest that ADE can occur in vivo, it does so under a restricted set of conditions that is modulated in part by C1q binding to individual IgG subclasses. In wild-type C1q-sufficient mice, ADE was observed at relatively low levels and only with mAbs of the mouse IgG$_1$ subclass, which poorly bind C1q. At present, it remains unclear why DII- or DIII-specific IgG$_1$ mAbs did not enhance infection more strongly. However, this small enhancement in vivo strictly depended on expression of activating Fc-γR. In contrast, in *C1q*$^{-/-}$ mice, robust ADE was observed with DII- or DIII-specific IgG$_{2a}$ mAbs; as these mAbs are predicted to bind C1q avidly, their inability to induce significant ADE in *C1q*$^{+/+}$ wild-type mice is consistent with their role in C1q-mediated inhibition of ADE. Surprisingly, the DIII-specific IgG$_{2b}$ mAb E16 did not promote significant ADE of WNV in *C1q*$^{-/-}$ mice, possibly due to the affinity of this IgG subclass for particular Fc-γRs expressed in the spleen. Consistent with this, mouse IgG$_1$ (E28 and E34) also did not strongly promote ADE in *C1q*$^{-/-}$ mice. Although further studies are required, the observation that IgG$_{2a}$, but not IgG$_1$ or IgG$_{2b}$, promotes ADE in *C1q*$^{-/-}$ mice is most consistent with an interaction with the Fc-γRI (CD64), which primarily binds monomeric IgG$_{2a}$ with high affinity (Hulett and Hogarth, 1994; Ravetch and Kinet, 1991).

Differences in ADE in vivo may also be modulated by the epitope specificity of individual antibodies. In vitro, virtually all mAbs that neutralize flavivirus infection also enhance infection in Fc-γR-expressing cells when used at subneutralizing concentrations (Morens et al., 1987; Pierson et al., 2007). Distinct mAbs show unique enhancement profiles in vitro as defined by their peak amplitude and breadth of enhancement of infection (Morens et al., 1987; Oliphant et al., 2006). In vivo, mouse IgG$_{2a}$ mAbs against the DIII-lateral ridge (E24) and DII-fusion loop (E18) epitopes consistently promoted ADE in *C1q*$^{-/-}$ mice over a relatively broad dose range. Surprisingly, human or mouse (IgG$_{2b}$) forms of E16, which also map to the DIII lateral ridge epitope (Nybakken et al., 2005; Oliphant et al., 2005), showed little capacity to enhance infection in wild-type or *C1q*$^{-/-}$ mice. At present, it remains unclear

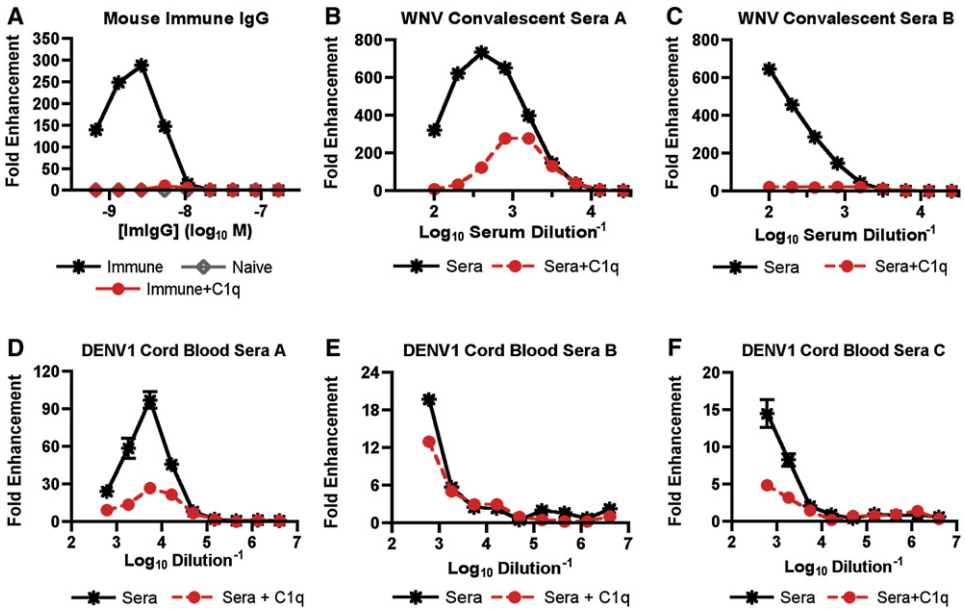

Figure 3. C1q Modulates ADE by Polyclonal Mouse and Human Antibody Obtained from WNV- and DENV-Infected Individuals

(A) IgG was purified from naive or WNV immune mouse serum by protein A affinity chromatography. Serial dilutions were mixed with WNV RVP in the presence or absence of C1q prior to infection of K562 cells. The data are expressed as the fold enhancement of infection compared to no antibody, and one representative experiment of three is shown.

(B–E) Heat-inactivated serum from WNV-infected human patients (B and C) or cord blood from DENV-immune mothers (D–F) was serially diluted and added to WNV or DENV1 RVP in the presence or absence of C1q prior to infection of K562 cells. The data are expressed as the fold enhancement of infection compared to no antibody. For (B), (D), and (F), one representative experiment of three is shown. Statistical analysis is described in the text. Error bars indicate standard deviations.

why only some mAbs that recognize closely overlapping epitopes enhance in vivo. Accordingly, we plan to repeat these studies with mAbs that bind a larger array of epitopes on the structural proteins prM and E.

Our in vivo enhancement studies are consistent with a recent publication that documented ADE of DENV in rhesus macaques after passive transfer of a humanized IgG_1 mAb (Goncalvez et al., 2007). As human IgG_1 is homologous to mouse IgG_{2a}, these isotypes interact with similar classes of Fc-γR (Hulett and Hogarth, 1994; Nimmerjahn et al., 2005; Ravetch and Kinet, 1991); one apparent difference, however, is that C1q suppresses ADE of mouse IgG_{2a} more completely than human IgG_1 (compare Figure 1D and Figure 2A). Although further investigation is warranted, this could contribute to the distinct ADE phenotypes in vivo in C1q-sufficient animals.

The dose of mouse E28 and E34 mAb that promoted in vivo enhancement in wild-type mice was surprisingly low (~1 ng per mouse) compared to that observed in K562 cells. Although we cannot explain with certainty the disparity between in vitro and in vivo findings, the following are three possible explanations. First, the mAbs appear to neutralize WNV more potently in vivo, inhibiting infection in the spleen at very low concentrations (~10 ng). As ADE occurs at subneutralizing concentrations of antibody, we would expect to see enhancement only at lower concentrations. Second, the Fc-γR that promotes antibody enhancement in the spleen may be different from our tissue culture model. Virtually nothing is known about the specific cell or Fc-γR that mediates ADE in vivo. Experiments are planned with specific *Fc-γR*$^{-/-}$ mice to directly address this question. Third, the tissue's levels of antibody are unknown, and specific IgG subclasses could accumulate differentially in lymphoid tissue because of antigen trapping by the high density of immune cells that express particular Fc-γR. Thus, the effective concentration of antibody in a given tissue compartment could be distinct.

Few previous studies have examined the interaction of complement with flaviviruses infection in detail. However, mice deficient in components of any of the three complement activation pathways (classical, lectin, or alternative) showed increased viral burden and lethality after WNV infection (Mehlhop and Diamond, 2006; Mehlhop et al., 2005). Complement also augmented antibody-mediated neutralization of WNV infection of BHK cells (Della-Porta and Westaway, 1977; Mehlhop et al., 2005). In terms of an association between complement and ADE, one group observed a serum and, presumably, complement-dependent ~10-fold enhancement of WNV infection by a non-neutralizing IgM antibody (Cardosa et al., 1983, 1986); although a link between complement, ADE, and IgG subclass was suggested, specific complement components and mAbs of individual IgG subclass were not examined. ADE has been suggested to contribute to the pathogenesis of several other viruses, including HIV and respiratory syncytial viruses (Fust et al., 1994; Gimenez et al., 1996; Homsy et al., 1990; Ponnuraj et al., 2001, 2003; Toth

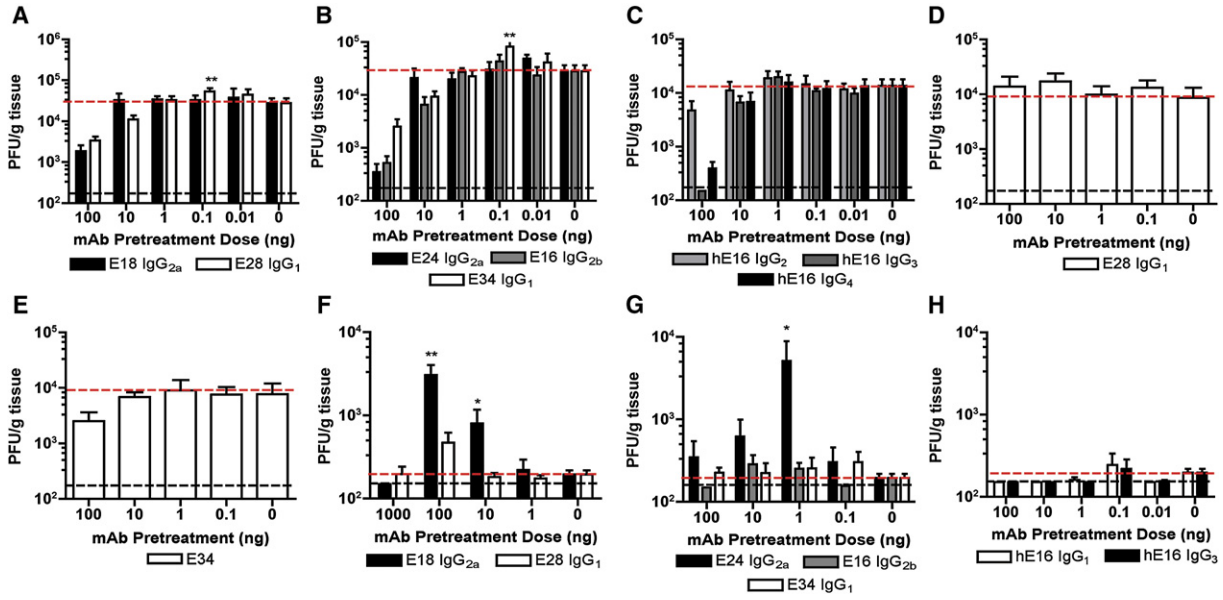

Figure 4. Effect of C1q and IgG Subclass on ADE In Vivo

Wild-type (A–C), *Fc-γR*$^{-/-}$ (D and E), or *C1q*$^{-/-}$ (F–H) C57BL/6 mice were pretreated with the indicated doses of anti-WNV mAbs (DII: E18 [mIgG$_{2a}$] and E28 [mIgG$_1$]; DIII: E16 [mIgG$_{2b}$, human IgG subclass variants], E24 [mIgG$_{2a}$], and E34 [mIgG$_1$]) 1 day prior to infection with 10^2 PFU of WNV. Four days later, spleens were harvested and homogenized, and virus infection was measured by plaque assay on BHK21 cells. Note at baseline, *C1q*$^{-/-}$ mice lack significant splenic infection because of the absence of an independent entry mechanism that requires C1q (Mehlhop and Diamond, 2006). The data are expressed as the number of plaque-forming units (PFU) per gram and reflect between four and eight mice per time point. Error bars indicate standard error of the means. Asterisks indicate differences that were statistically significant ($^*p < 0.05$ and $^{**}p < 0.005$). The dotted line indicates the limit of sensitivity of the assay.

et al., 1991). Here, we show that C1q restricts ADE by anti-flavivirus antibodies in an IgG subclass-specific manner. Consistent with our data, a recent study with measles virus showed complement-dependent suppression of ADE in vitro by a single mAb (Iankov et al., 2006); however, no analysis of the specific complement components or IgG subclass was performed.

At present, on a cellular level, we do not know the precise mechanism by which C1q minimizes ADE. Part of the difficulty in answering this question is that it remains unclear exactly how antibody enhances virus infection. Although classical biochemical and microscopic studies (Gollins and Porterfield, 1984, 1985) suggest that virus opsonization by subneutralizing concentrations of antibodies enhances cell attachment via a Fc-γR-dependent mechanism, recent experiments indicate that active Fc-γR signaling with changes in intracellular innate responses may contribute to enhanced infection. For example, signaling-competent and -incompetent forms of Fc-γRI and Fc-γRII induced differential enhancement of DENV immune complex infectivity (Rodrigo et al., 2006). In addition, ADE reduced antiviral gene transcription through STAT-1 and NF-κB in macrophages, effectively increasing the permissiveness of the cell for viral infection (Mahalingam and Lidbury, 2002). C1q restriction of ADE could limit virus attachment to cells by directly blocking Fc-γR binding to the Fc moiety on the antibody heavy chain. This appears plausible, as C1q is a large multimeric protein (Kishore and Reid, 2000) and the C1q and Fc-γR binding sites are proximal (Idusogie et al., 2000, 2001). Alternatively, C1q could inhibit ADE independently by attenuating Fc-γR signaling or internalization or directly restricting structural movements of the envelope protein that are required for viral fusion (Modis et al., 2004).

Although our experiments demonstrated ADE of WNV infection in vivo in *C1q*$^{-/-}$ mice, we did not observe a significant change in disease phenotype or survival. This may be due to one of several possibilities. First, WNV infection via a subcutaneous route has a very flat dose-response curve in C57BL/6 mice such that inoculation of 10^2 and inoculation of 10^6 plaque-forming units (PFU) have virtually the same lethality (Diamond et al., 2003a); thus, increased viral burden in the spleen does not directly lead to enhanced dissemination to the brain and spinal cord. Second, the linkage between ADE and severe disease as postulated for DENV infection may not occur for WNV because one or more additional steps of viral pathogenesis are absent in the life cycle of WNV. In support of this possibility, an enhanced risk of severe WNV disease during secondary infection or vaccine challenge has never been described. Instead, ADE and severe disease may be more significant for other flaviviruses, such as DENV. Despite much effort, an adequate small animal model of ADE and DHF/DSS is lacking. As our data establish that C1q also modulates ADE by DENV infection, we plan to use *C1q*$^{-/-}$ mice and IgG$_{2a}$ mAbs along with specific adapted DENV strains (Shresta et al., 2006) to evaluate the link between ADE and pathogenesis.

Our studies with C1q, flaviviruses, and ADE may help explain the increased occurrence of DHF/DSS in infants. Primary DENV infection of infants from DENV-immune mothers results in DHF/DSS at a higher frequency than expected (Halstead et al., 2002; Nguyen et al., 2004). This is believed to occur as passively transferred anti-DENV antibody wanes and binds to virus in a manner that is inadequate for neutralization but sufficient for enhancement (Halstead et al., 2002). Although human IgG_1 antibodies are enriched, anti-DENV antibodies of all IgG subclasses are present in cord and naive infant blood samples (Wataveeradej et al., 2003), anti-DENV IgG_4 antibodies are higher in patients with DSS (Koraka et al., 2001), and significant variation in ADE was observed among patient serum in the presence of C1q (see Figure 3). Infant ADE may also occur more frequently as serum C1q levels are 33% to 55% lower in the first year of life (Davis et al., 1979). Thus, the potential for ADE and DHF in infants with DENV-immune mothers at 6–9 months may be defined by waning neutralizing titers, IgG subclass specificity against a particular DENV, and depressed systemic C1q levels. As DHF is primarily a disease of the developing world, it is intriguing to consider that IgG subclass skewing could result from circulating T_H2 cytokines that are present because of coinfection with parasites (Maizels, 2005). Finally, our experiments may explain why individuals homozygous for the Fc-γRIIa-131^H allele are more susceptible to DHF (Loke et al., 2002): these variant Fc-γRIIa bind the poorly C1q-fixing hu-IgG_2 immune complexes with higher affinity than the Fc-γRIIa-131^R allele.

Despite the large number of individuals with circulating anti-DENV antibodies who become secondarily infected with a distinct serotype, few (~1/200) develop DHF/DSS. The enhancing activity of maternal or preillness serum has had conflicting prognostic utility (Kliks et al., 1988, 1989; Laoprasopwattana et al., 2005) for severe DENV disease. Although more studies are required, addition of C1q to virus enhancement assays may improve their predictive value. Finally, our data suggest that development of adjuvants that favorably skew IgG subclass responses may be important for vaccines against viruses in which ADE is a concern.

EXPERIMENTAL PROCEDURES

Cells and Viruses

Vero, K562, and a Raji cell line stably expressing DC-SIGNR were maintained as described (Pierson et al., 2006, 2007). Mouse bone marrow-derived macrophages were generated and maintained as previously described (Samuel et al., 2006). Infections were performed with WNV RVP that were produced using a previously described complementation strategy (Pierson et al., 2006). The procedures for generating DENV1 RVP will be described in greater detail elsewhere (C.A.-S. and T.C.P., unpublished data). The infectious WNV (3000.0259) strain was described previously (Ebel et al., 2001).

Mouse Serum and Complement

Blood was collected by axillary venupuncture into serum separator tubes (Sarsted, Newton, NC) from 8- to 12-week-old male C57BL/6 wild-type, *C3*$^{-/-}$ (Circolo et al., 1999), and *C1q*$^{-/-}$ (Botto et al., 1998) mice that were obtained commercially (Jackson Laboratories, Bar Harbor, ME) and from colleagues (*C1q*$^{-/-}$, M. Botto, London, UK; *C3*$^{-/-}$, H. Molina, St Louis, MO). Blood was clotted on ice and serum was pooled, aliquoted, and frozen at −80°C until use. Heat-inactivated serum was treated at 56°C for 30 min. Purified human C1q was obtained commercially (Advanced Research Technologies, Tyler, TX) and stored aliquoted at −80°C.

In Vitro Enhancement Assays

The enhancing activity of the WNV-reactive mAbs in the presence or absence of 5% mouse serum or purified C1q (50 μg/ml) was determined with K562 cells in at least three independent experiments in triplicate using a high-throughput flow cytometry-based assay as described (Pierson et al., 2006, 2007). Antibody enhancement of WNV infection was also determined with bone marrow-derived macrophages. WNV was preincubated with serial dilutions of antibody in the presence or absence of mouse serum or purified C1q. Adherent macrophages were infected with opsonized WNV, washed, and cultured an additional 24 hr. Supernatants were collected and assayed for production of infectious WNV by viral plaque assay on BHK21 cells (Diamond et al., 2003a).

E16 Human IgG Subclass Switch Variants

Human IgG subclass switch variants of humanized E16 IgG_1 (Oliphant et al., 2005) were constructed by inserting the humanized E16 V_H region as an NheI-ApaI fragment into respective pCI-neo-derived vectors containing either the human IgG_2, IgG_3, or IgG_4 constant region cDNA. The resulting plasmids were cotransfected with humanized E16 light chain (also in pCI-neo) into HEK293 cells using Lipofectamine 2000 (Invitrogen) for production of each IgG subclass switch variant. IgG of different subclasses were purified from cell supernatants using MabSelect protein A Sepharose (GE Healthcare) and size exclusion chromatography. Concentrations were determined by OD_{280} measurements.

ADE In Vivo

All mice were housed in a pathogen-free mouse facility at Washington University School of Medicine. Studies were performed in compliance with the guidelines of the Washington University School of Medicine Animal Safety Committee. All infections used a low-passage WNV isolate 3000.0259 that was propagated once in C6/36 *Aedes albopictus* cells. Eight- to twelve-week-old *C1q*$^{-/-}$, Fc-γR I, III, and *IV*$^{-/-}$ (which lack the common signaling γ-chain), and wild-type C57BL/6 mice were passively transferred mAb by intraperitoneal injection at day −1 and then infected via footpad with 10^2 PFU of WNV on day 0. Four days after infection, spleens were removed, weighed, homogenized using a bead beater apparatus (BioSpec Products, Inc), and titrated for virus by plaque assay on BHK21 cells as described (Diamond et al., 2003a).

Statistical Analysis

For in vitro experiments, a paired Student's t test was used to determine statistically significant differences. For viral burden analysis, differences in titers were analyzed by the Mann-Whitney test. All data were analyzed using Prism software (GraphPadPrism4, San Diego, CA).

Supplemental Data

The Supplemental Data include two supplemental figures and can be found with this article online at http://www.cellhostandmicrobe.com/cgi/content/full/2/6/417/DC1/.

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