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REVIEW

The Antigenic Structure of Zika Virus and Its Relation to Other Flaviviruses: Implications for Infection and Immunoprophylaxis

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SUMMARY Zika virus was discovered ~70 years ago in Uganda and maintained a low profile as a human disease agent in Africa and Asia. Only recently has it caused explosive outbreaks in previously unaffected regions, first in Oceania and then in the Americas since 2015. Of special concern is the newly identified link between congenital malformations (especially microcephaly) and Zika virus infections during pregnancy. At present, it is unclear whether Zika virus changed its pathogenicity or whether the huge number of infections allowed the recognition of a previously cryptic pathogenic property. The purpose of this review is to discuss recent data on the molecular antigenic structure of Zika virus in the context of antibody-mediated neutralization and antibody-dependent enhancement (ADE) of infection, a phenomenon that has been implicated in the development of severe disease caused by the related dengue viruses. Emphasis is given to epitopes of antibodies that potently neutralize Zika virus and also to epitopes that provide antigenic links to other important human-pathogenic flaviviruses such as dengue, yellow fever, West Nile, Japanese encephalitis, and tick-borne encephalitis viruses. The antigenic cross talk between Zika and dengue viruses appears to be of special importance, since they cocirculate in many regions of endemicity and sequential infections are likely to occur frequently. New insights into the molecular antigenic structure of Zika virus and flaviviruses in general have provided the

foundation for great progress made in developing Zika virus vaccines and antibodies for passive immunization.

KEYWORDS flavivirus antigenic structure, Zika virus

INTRODUCTION

For several decades, Zika virus (ZIKV) was considered an insignificant member of the genus *Flavivirus* of the family *Flaviviridae* (1, 2). This genus comprises about 70 different viruses, including some of the most important human-pathogenic arthropod-borne viruses, such as yellow fever (YF), dengue (Den), West Nile (WN), Japanese encephalitis (JE), and tick-borne encephalitis (TBE) viruses (1). Human Zika virus infections were reported infrequently in Africa and Asia and associated with mild febrile symptoms but were not linked to serious disease. The situation changed dramatically when Zika virus started to cause massive outbreaks in Oceania, first in Yap island (2007) and since 2013 in many other Pacific islands, including French Polynesia, New Caledonia, the Cook Islands, and Easter Island (3, 4). From there, the virus continued to spread eastwards, and in 2015, it emerged as a new pathogen in the Americas (3, 4). According to the WHO, up to 4 million infections may occur in 2016 in the Americas alone (5). Of the greatest concern is the newly discovered causal association between microcephaly as well as other congenital malformations and Zika virus infections during pregnancy (reviewed in reference 6). In addition, Zika virus infections have been linked to neurological disorders such as Guillain-Barré syndrome (GBS) (7). Increasing numbers of autochthonous Zika virus infections are now also being reported in Southeast Asia and Africa, which are caused by local strains (8–10). Increased awareness and surveillance as a result of the American outbreak may have contributed to their specific diagnosis.

Importantly, Zika virus can use humans as the only vertebrate host and maintain human-to-human transmission cycles through anthropophilic *Aedes* mosquitoes, similar to dengue, YF, and Chikungunya viruses (11). *Aedes aegypti* appears to be the major culprit of causing explosive epidemics with these viruses because it not only lives in close association with humans but also feeds almost exclusively on humans, whereas other competent mosquito species do not have such a human-restricted feeding behavior (11). Of note, ZIKV can also be transmitted sexually and currently appears to be the only known arthropod-borne virus (arbovirus) linked to this property in humans (12). The extent to which this mode of transmission contributes to the observed spread of Zika virus and congenital malformations remains to be elucidated (13, 14).

Modeling studies estimate that up to 2.17 billion people live in regions conducive to Zika virus transmission (15). This would include large parts of North America, where *Aedes aegypti* and/or *Aedes albopictus* mosquitoes are prevalent (16). The presence of competent vectors is certainly not the only factor required for virus spread, but it is of concern that autochthonous human transmission has been identified in Florida (17, 18), and a case reported in Texas is under investigation (https://www.cdc.gov/media/releases/2016/p1128-zika-texas.html). As a result of global travel and trade, human Zika virus infections originating in areas of endemicity are constantly exported to virtually every part of the world (19, 20) and may become the source of local transmission when appropriate conditions prevail. It is currently unclear whether Zika virus will be able to establish sylvatic transmission cycles involving nonhuman primates (like YF and dengue viruses) and whether such enzootic cycles may be necessary for Zika virus to remain endemic in the long run (21, 22).

The recent spread and newly observed associations with congenital infections as well as sexual transmission urge the question of whether specific mutations had occurred that made the virus change from an agent that slumbered in some regions of Africa and Asia and caused only relatively mild disease in rare cases to an emerging outbreak virus with disconcerting pathology. Unfortunately, no conclusive evidence for causal genetic links could be obtained so far from extensive sequence comparisons performed with old and new virus strains, although a number of mutations were identified in contemporary outbreak strains compared to older Asian and African strains

(23). Concrete associations between specific mutations and biological properties therefore remain to be elucidated by complementary technologies such as the use of reverse genetic systems in combination with biological analyses (24, 25) and relevant animal models (reviewed in reference 6).

All flaviviruses are antigenically related to various degrees, and immunological cross-reactions have been implicated not only in cross-protection but, under certain conditions, also in infection enhancement phenomena that may exacerbate disease in humans and/or facilitate vector transmission (21). This is of special relevance in regions where different flaviviruses cocirculate, allowing sequential infections to occur on the background of immunity to an antigenically related flavivirus. Cocirculation is found in many parts of the world and specifically also in regions where Zika virus recently emerged (1).

Antibodies have been shown to be important for long-term protection against flavivirus disease after both natural infection and vaccination (26) but may also play a key role in infection enhancement under certain conditions that are not yet fully understood (antibody-dependent enhancement [ADE]) (27). In this context, it has been hypothesized that congenital Zika virus infections could be facilitated by ADE phenomena mediated through preexisting cross-reactive but nonneutralizing antibodies, such as those induced by dengue virus infections, which are highly prevalent in the new regions where Zika virus is endemic (27). Cross-reactive antibodies can form infectious virus-antibody complexes that are efficiently endocytosed by Fcγ receptor-positive cells (monocytes, macrophages, and dendritic cells), leading to strongly enhanced virus production (28) (see Antibody-Dependent Enhancement of Zika Virus Infection, below). In addition to Fcγ receptor-mediated effects that can increase viral loads, it has also been speculated that neonatal Fc (Fcn) receptors could play a role in transplacental infections by mediating the transcytosis of infectious virus-antibody complexes through the placental barrier (6, 29, 30). However, the mechanism of transcytosis (31) would expose the virus to the acidic pH in endosomes (where Fcn receptors reside [31]), which might cause irreversible conformational changes of viral envelope proteins and virus inactivation (see Flavivirus Structure and Life Cycle, below). So far, there is no experimental evidence that Zika virus can use Fcn receptors for crossing the placenta.

We review here the antigenic landscape of Zika virus in comparison to other flaviviruses in the context of antibody-mediated virus neutralization and infection enhancement and discuss details of viral particle structures that are relevant for understanding antibody cross-reactions and their possible implications for immunopathogenesis as well as immunoprophylaxis.

FLAVIVIRUS STRUCTURE AND LIFE CYCLE

Flaviviruses are simple enveloped viruses containing a positive-stranded genomic RNA of ~11 kb associated with a capsid protein (C) in a structurally ill-defined isometric capsid (Fig. 1A). This capsid is surrounded by a lipid membrane with two integrated proteins: E (envelope) and prM (precursor of membrane [M]) in immature particles (Fig. 1A, left) and E and M in mature particles (Fig. 1A, right).

Structural information on viral envelopes was obtained by X-ray crystallography (32–39) and cryo-electron microscopy (EM) (40–48) with a limited set of flaviviruses (mostly Den, WN, JE, and TBE viruses) and is illustrated in Fig. 1B, C, and F for dengue viruses. Immature virions are spiky, have a diameter of ca. 60 nm, and display 60 projections of trimers of prM-E heterodimers (Fig. 1B). Mature virions, in contrast, are somewhat smaller (diameter of 50 nm) and have a smooth surface (Fig. 1C), which is formed by a herringbone-like array of 90 E protein homodimers oriented parallel to the viral membrane (Fig. 1 D). Details of the dengue virus E protein in mature virions are shown in its side and top views in Fig. 1F. The C terminus of E is anchored to the viral membrane by two transmembrane (TM) helices that are followed by the so-called stem region, which also includes short helices, one of which is partially embedded in the viral membrane (42, 46, 47).

The top view of E (Fig. 1F, bottom) reveals its antiparallel dimeric structure and the

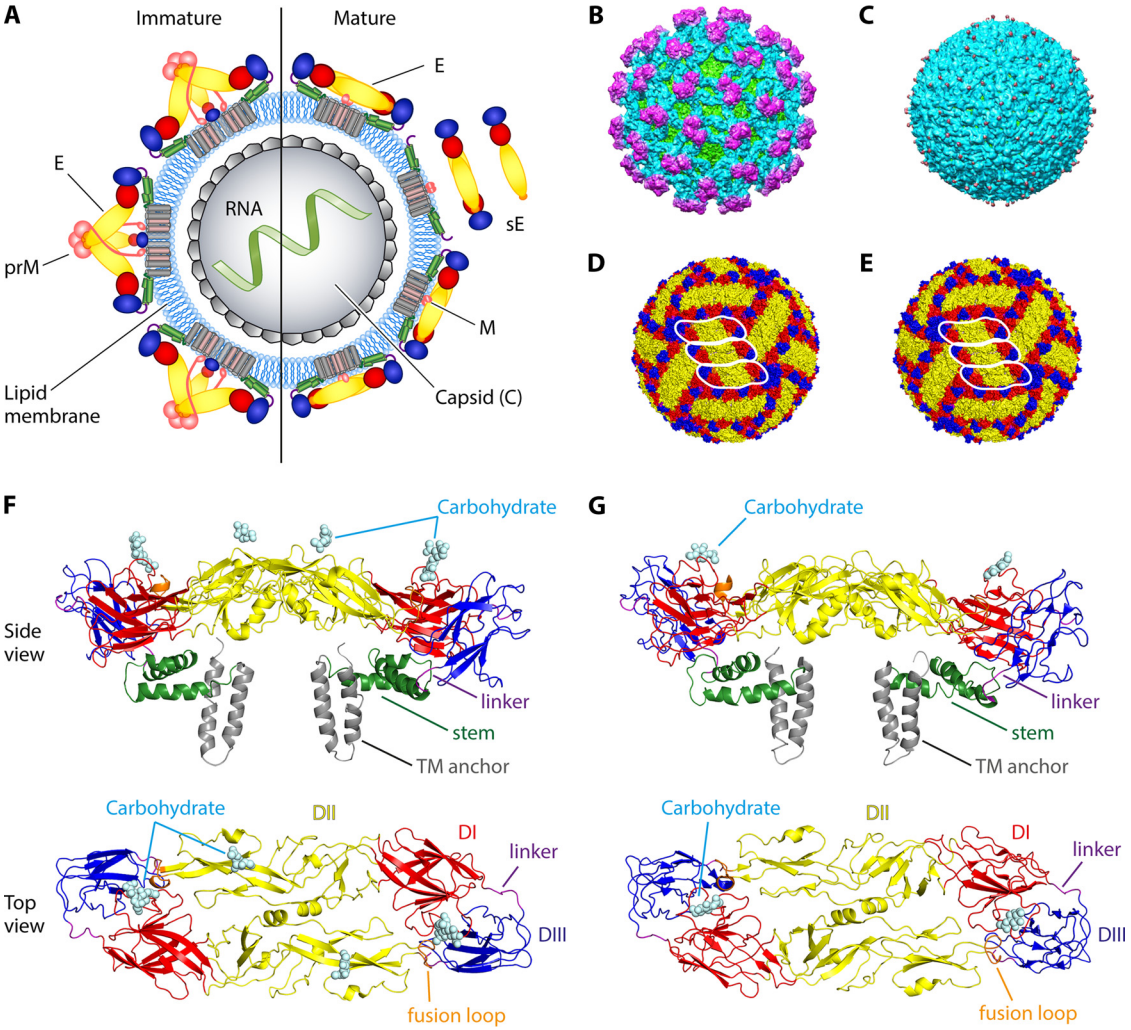

FIG 1 Schematics and structures of flavivirus particles and E proteins. (A) Schematic representation of immature (left) and mature (right) virions. C, capsid protein; prM, precursor of membrane protein (M); E, envelope protein; sE, soluble envelope protein. (B and C) Cryo-EM structures of immature and mature dengue 1 virus, respectively (reprinted from reference 47 with permission). (D and E) Surface representations of the herringbone arrangement of E dimers at the surface of mature dengue (D) and Zika (E) viruses. Images were constructed by using data reported under PDB accession numbers 3J27 and 5IZ7 (46, 64). A set of 3 parallel dimers forming one of the 30 "rafts" in the herringbone structure is highlighted by white contours. (F and G) Ribbon diagrams of the dengue 2 and Zika virus E protein dimers in their side and top views, respectively. Images were constructed by using data reported under PDB accession numbers 3J27 and 5IZ7 (46, 64). Asn-linked carbohydrates are shown as light blue spheres. TM, transmembrane. In all representations, the three E protein domains are displayed in red (DI), yellow (DII), and blue (DIII), and the DI-DIII as well as DIII-stem linkers are shown in purple. The fusion loop at the tip of domain II is shown in orange.

three domains (domain I [DI], DII, and DIII) that are connected to the TM anchor and stem regions by a short linker (Fig. 1F, top). This representation also highlights the fusion loop (FL) at the tip of DII, which is the most conserved structural element among all flavivirus E proteins. In the E dimer, it is buried by interdigitation with a hydrophobic pocket provided by domains I and III of the second subunit but becomes exposed after virus entry into cells to initiate membrane fusion (Fig. 2 and see below). As described in more detail below, the FL is responsible for the broad antibody cross-reactivity observed among all flaviviruses and plays an important role as an antigenic site that contributes to antibody-mediated enhancement of infection.

Flaviviruses undergo a number of structural changes that control and drive specific functions in different phases of the viral life cycle, including virus assembly in infected cells and entry into new cells (Fig. 2A). Virion assembly takes place in the endoplasmic reticulum (ER), involving an incompletely defined budding process that leads to the formation of immature, noninfectious particles with prM-E heterodimers in their enve-

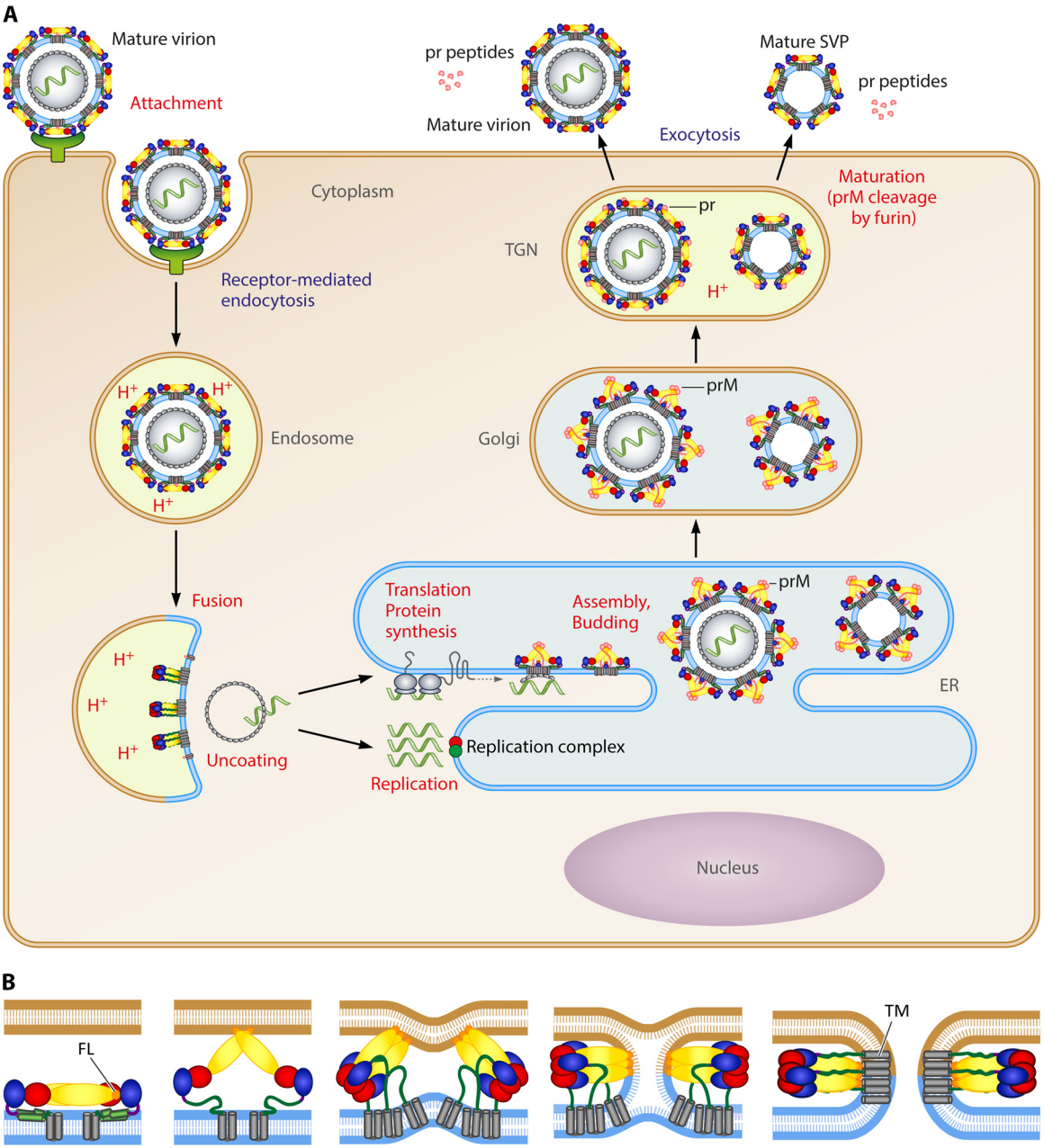

FIG 2 Flavivirus life cycle and fusion mechanism. (A) The virus enters cells by receptor-mediated endocytosis and fuses its membrane by an acidic-pH-triggered mechanism in the endosome to release the viral RNA. The positive-stranded genomic RNA serves as the only viral mRNA and leads to the synthesis of a polyprotein that is co- and posttranslationally processed into three structural and seven nonstructural proteins. Virus assembly takes place at the ER membrane and leads to the formation of immature virions, which are further transported through the exocytic pathway. The acidic pH in the TGN causes structural changes that allow the cleavage of prM by the cellular protease furin and lead to the herringbone-like arrangement of E (Fig. 1D and E). At acidic pH, the cleaved pr part remains associated with the particles (preventing premature fusion in the TGN) and falls off at the neutral pH of the extracellular environment. Subviral particles (SVPs) are formed as a by-product of virion assembly and contain a lipid membrane and prM-E complexes but lack a capsid. SVPs are transported, processed, and released like whole virions. (B) Schematic of flavivirus membrane fusion. The acidic pH in the endosome causes the dissociation of the E dimer and exposure of the FL, which mediates the interaction with endosomal membranes. Further structural changes lead to the relocation of DIII and the formation of a trimer in a hairpin-like structure in which the FL and the TM regions are juxtaposed. These rearrangements provide the energy for membrane fusion and result in the merger of the two lipid bilayers. Color codes are as described in the legend of Fig. 1.

lopes (Fig. 1A, left, and 2A) (49). They are transported through the exocytic pathway of the cell until they reach the *trans*-Golgi network (TGN). Here, the slightly acidic pH triggers conformational changes that lead to a reorganization of the envelope proteins and the cleavage of prM into pr and M by the cellular protease furin (45, 50). The

cleaved pr fragment remains associated with the particle at the acidic pH of the TGN but dissociates at the neutral pH of the extracellular environment (45, 51), thus generating mature infectious virions in which E has adopted a metastable conformation. The efficiencies of the cleavage of prM can vary among flaviviruses and viruses grown in different cell types, leading to the release of partially mature particles (see "Partially Mature Virions," below).

As natural by-products of virus assembly, subviral particles (SVPs) that contain only a lipid bilayer and the membrane proteins but that lack a capsid are formed (Fig. 2) (reviewed in reference 52). They bud from the ER membrane by lateral interactions between prM and E only and are transported, cleaved by furin, and released from the cells analogously to whole virions. Since the SVPs display E dimers in a virus-like conformation and can also be produced in a recombinant form by the coexpression of prM and E, they are prime candidates for particulate subunit vaccines or DNA vaccines that generate SVPs (see "Zika Vaccines," below).

Virus entry occurs by receptor-mediated endocytosis, and E has been implicated in cell attachment and mediates endosomal membrane fusion (Fig. 2). A plethora of possible receptor molecules have been described in the literature (for reviews, see references 53 and 54), and their engagement in attachment and/or internalization may differ in the various hosts and tissues that flaviviruses encounter in their complex ecological cycles. Despite intensive research, the contribution of specific receptors to flavivirus entry in different types of relevant target cells in different hosts remains incompletely understood. Recent studies indicated that proteins of the TIM (T cell immunoglobulin mucin domain) and TAM (Tyro3, Axl, and Mer) receptor families can mediate the entry of flaviviruses and specifically also Zika virus into cells (53–55). They do not interact with E but interact with lipids exposed in the viral membrane. The normal function of these proteins is to recognize externalized phosphatidylserine on apoptotic cellular debris and to trigger its endocytic engulfment by phagocytic cells. Hijacking this process by viruses has therefore been termed apoptotic mimicry (56, 57). There is strong evidence that Zika virus may use this mechanism to gain access to different tissues throughout the human body. While the TAM receptor Axl was proposed to play a critical role for virus entry into human skin fibroblasts (55), endothelial cells (58), and brain cells (59, 60), TIM1 appeared to dominate as an entry factor in infection of placental tissues (61). In any case, the fact that Zika virus infection can be mediated by interactions with TIM/TAM receptors indicates that parts of the lipid membrane are accessible for such ligand interactions at the surface of infectious particles. This might be the case at the immature side of partially mature viruses (see "Partially Mature Virions," below), and even the seemingly closed shells of fully mature viruses may transiently expose the lipid membrane due to dynamic motions of envelope proteins at the viral surface (see "Virus Breathing," below).

Membrane fusion is triggered by the acidic pH in endosomes and driven by an irreversible structural change that converts E from a metastable dimer into a stable trimer (Fig. 2B). In this postfusion conformation, E adopts a hairpin-like structure by the relocation of DIII and the "zippering" of the stem along DII in the trimer, resulting in the juxtaposition of the FL and the TM helices in the fused membrane (Fig. 2) (62). The metastability of E and its sensitivity to low pH explain the function of prM during the exocytosis of immature virions, which is to stabilize E against the acidic pH in the TGN and thus to prevent premature fusion already at this stage of the viral life cycle (51). All of the transitions during assembly, maturation, and entry require substantial structural flexibility of E, especially at the junctions between its domains (Fig. 1F, top view), which are likely to contribute to the dynamic motions observed in flavivirus envelopes (see "Virus Breathing," below).

Structure of Zika Virus

The overall architecture of Zika virus is similar to that of other flaviviruses, as revealed by cryo-EM of purified virions (63, 64) and X-ray crystallography of recombinant envelope proteins either alone or in complex with fragments of monoclonal

antibodies (MAbs) (65, 66). The comparison with dengue virus in Fig. 1F and G shows that all of the essential structural features are conserved, including the domain organization of E and its antiparallel orientation in homodimers as well as the herringbone-like arrangement of these dimers at the surface of mature virions (Fig. 1D and E).

Similarly to most other flaviviruses, there is a single glycan attached to a loop in domain I of the Zika virus E protein that has been highlighted as a noticeable feature and hypothesized to govern cellular tropism or contribute to disease outcomes (63). In contemporary Asian and American strains and all other strains since the outbreak in Yap island 2007, this glycan loop is longer than those in YF, dengue, and TBE viruses but similar to those in WN and JE viruses. Of note, many older Zika virus strains have a deletion of the loop or a mutation at the carbohydrate attachment site (N_{154}DT to N_{154}DI) that would lead to nonglycosylated E proteins (67, 68). This lack of glycosylation is specifically observed in African strains isolated before 2007 (68), including the first Ugandan isolate from 1947 (MR766-UGA-1947). It is striking that individual data bank entries corresponding to the same strain in principle (e.g., of MR766-UGA-1947, DAK-41524-1984, or P6-740-MYS-1966) contain different versions of deletions or glycan-abolishing mutations without a deletion, raising the question of whether the loss of glycosylation might be a consequence of virus passage in the laboratory. This may indicate that the carbohydrate is essential for maintaining the virus in its natural ecological cycle but not for replication under certain experimental conditions. The issue of glycosylation is also of practical relevance because it may affect the results of neutralization assays and contribute to strain-specific variations found in such analyses (see "Zika Virus as a Single Serotype," below).

STRUCTURAL BASIS OF FLAVIVIRUS NEUTRALIZATION

Binding of antibodies to E can interfere with receptor interactions and/or membrane fusion (Fig. 2A), making this protein the primary target of virus-neutralizing antibodies. Many studies suggest that neutralizing antibodies are the key mediators of long-term protection after flavivirus infection and vaccination (26). The surface of the virion is likely to represent a continuous antigenic landscape of potential epitopes that can have different structural complexities. These epitopes may encompass residues from individual domains only (65, 69–74), be located at the junction between domains in the E monomer (75), or represent more complex quaternary structures that are dependent on the E dimer (66, 76, 77) or even its herringbone arrangement at the virus surface (78–80). The epitopes that dominate an antibody response may differ between different flaviviruses (81, 82), different species (83, 84), and different individuals of the same species (85, 86).

Model studies with West Nile virus have shown that flavivirus neutralization is a "multihit" phenomenon that requires that the number of antibodies bound to the virus exceeds a certain threshold (87). Whether an antibody can reach this critical threshold depends on its affinity and the accessibility of its epitope on the virion surface (82). Mechanisms of antibody-mediated virus neutralization may include inhibition of virus attachment to cell receptors, a block of internalization, or interference with the conformational changes required for membrane fusion but are likely to be a composite of such mechanisms (88).

Importantly, virus neutralization and other antibody-mediated phenomena, such as enhancement of infection, cannot be understood completely on the basis of the compact and homogeneous structures of mature viruses, as displayed in Fig. 1A and C to E. There are two important features that add substantially to the complexity of virus-antibody interactions and modulate their biological consequences: (i) incomplete maturation cleavage of prM resulting in the generation of virions that are only partially mature and (ii) dynamic motions of viral particles ("virus breathing") leading to the transient exposure of seemingly cryptic protein surfaces that can be recognized by antibodies.

Partially Mature Virions

It is a typical feature of flaviviruses in general that the maturation cleavage of prM can be incomplete, and substantial variability between viruses produced in different cells and even between different strains of the same virus has been observed (89). In many instances, therefore, virus preparations are heterogeneous mixtures of immature, mature, and partially mature viral particles (90, 91). The cryo-EM reconstructions of Zika virus (Fig. 1E) should be regarded as idealized structures, because they were based on viruses produced in Vero cells overexpressing furin to ensure a homogeneous mature virus preparation (63) or in insect cells that apparently also secrete largely mature virions (64).

As shown for dengue virus, partially mature flaviviruses display a mosaic structure composed of variably sized surface regions in their immature and mature conformations (92). Importantly, such particles are infectious because a limited number of E proteins in their metastable, mature conformation is apparently sufficient to mediate viral membrane fusion and other entry functions (93). Completely immature particles can also become infectious when they are internalized by cells and their prM proteins are proteolytically cleaved by furin-like proteases present in endosomes (94).

The maturation state of flaviviruses can have strong biological implications, including influences on the effects of antibody binding, such as virus neutralization and antibody-dependent enhancement of infection. In many instances, incomplete cleavage results in more efficient neutralization because of the increased accessibility of certain epitopes in partially mature viruses (95). This effect can contribute to cell type-dependent differences of neutralizing antibody titers when viruses produced in different cells and containing different amounts of prM are used in such analyses (95–97). A certain degree of assay standardization can be achieved by using furin-overexpressing cells, which produce completely mature viruses (98). The role of partially mature particles in the enhancement of infection may be due to antibodies recognizing epitopes in E that are modulated through the presence of prM but also to antibodies directed directly to prM (91, 99, 100). These issues are discussed in more detail in Antibody-Dependent Enhancement of Zika Virus Infection, below.

Although variation of prM cleavage has been described for flaviviruses grown in cell culture, there is currently no information for any flavivirus regarding its maturation status in natural hosts (including humans) and the different tissues infected in these hosts, let alone clues of possible influences of the extent of maturation cleavage on transmission and/or pathogenesis. These are important unresolved questions, not only because of possible direct implications of varying prM cleavage on biological properties of the viruses but also because of indirect effects resulting from the induction of anti-prM antibodies. These antibodies usually have only very low neutralizing potency, if at all, but can contribute strongly to the enhancement of infection (100, 101).

Virus Breathing

Although cryo-EM structures have suggested that flavivirus envelopes resemble closed shells, there is now convincing evidence that the proteins in these envelopes, in both mature and immature particles, are in dynamic motion and continuously sample different conformations (102). This became apparent from studies of virus neutralization by antibodies that target epitopes that would be inaccessible in static models of virion structure (70, 72, 99, 103). This phenomenon, usually referred to as virus breathing, seems to be a consequence of the conformational flexibility of E required in the different transition stages of the viral life cycle (Fig. 2A and B).

Some conformational changes during virus breathing can be irreversible and lead to virus inactivation, referred to as "intrinsic decay" (103), which can differ strongly among flaviviruses. In a recent study, the thermal resistance of Zika virus infectivity was found to be substantially higher than that of dengue 2 and dengue 4 viruses, indicative of a comparatively low degree of intrinsic decay and the high stability of at least the Zika virus strain (H/PF/2013) used in these experiments (64). More extensive analyses on

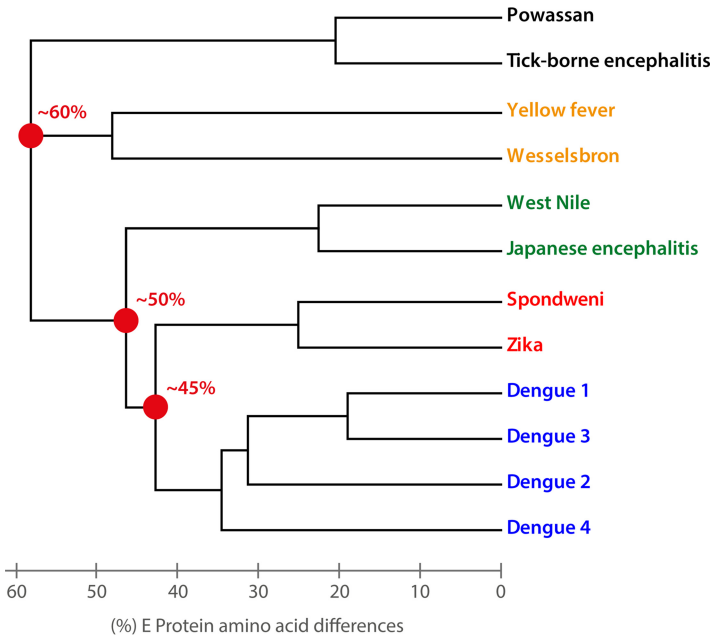

FIG 3 Relationships of flaviviruses based on percent amino acid sequence divergence of E proteins. Sequences were obtained from the ViPR data bank (https://www.viprbrc.org/). GenBank accession numbers are as follows: L06436 for Powassan virus, U27495 for tick-borne encephalitis virus, AY640589 for yellow fever virus, JN226796 for Wesselsbron virus, DQ211652 for West Nile virus, D90194 for Japanese encephalitis virus, DQ859064 for Spondweni virus, KJ776791 for Zika virus, AF226687 for dengue 1 virus, DQ863638 for dengue 3 virus, M29095 for dengue 2 virus, and GQ398256 for dengue 4 virus.

flavivirus stability revealed that Zika virus is not uniquely stable (104). Also, this study, however, demonstrated a substantially higher stability of Zika than of dengue virus.

The role of virus breathing, stability, and intrinsic decay in the natural ecological cycles and/or human pathogenicity of different flaviviruses is incompletely resolved, similar to the situation with partially mature virions. However, several lines of experimental evidence suggest a substantial impact of breathing on biological properties. First, breathing can expose the lipid membrane even in completely mature particles (105–107), which is required for interactions with TIM/TAM receptors, as described in Flavivirus Structure and Life Cycle, above. Second, the dynamics of the flavivirus envelopes can allow antibody binding to seemingly cryptic epitopes, which may result in either virus neutralization or enhancement of infection. Importantly, the neutralizing potency of certain antibodies to cryptic epitopes may be strongly affected by strain-specific mutations that do not change the epitope itself but affect conformational dynamics and thus can increase epitope accessibility (108). Such effects underscore the complexity of the antigenic landscape of flaviviruses and, like the issue of prM cleavage, are important to be considered in the standardization of virus neutralization assays (103).

ANTIGENIC RELATIONSHIPS OF ZIKA VIRUS AND OTHER FLAVIVIRUSES

Flavivirus Serocomplexes

Although flavivirus E proteins have a highly conserved structural organization (Fig. 1F and G), up to 60% of the amino acids can differ between distantly related mosquito- and tick-transmitted viruses within the genus. Figure 3 displays the amino acid sequence divergence of the most important human-pathogenic flaviviruses, together with some close relatives that are considered to be members of the same antigenic subgroup. Traditionally, flaviviruses have been subdivided into so-called serocomplexes, comprising members that are cross-neutralized by polyclonal sera. Cross-neutralization between different serocomplexes is usually not observed (109). Such

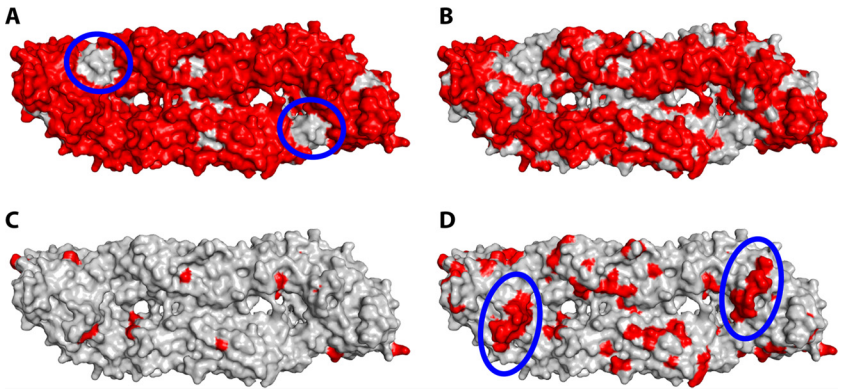

FIG 4 Display of variable surface-exposed amino acids highlighted in red on the background of the Zika virus E dimer (gray). (A) All amino acids that differ in a comparison between Zika virus and the other mosquito-borne flaviviruses displayed in Fig. 3. Circles highlight the conserved patch of amino acids around the fusion loop. (B) All amino acids that differ in a comparison between Zika virus and dengue viruses 1 to 4. (C) Amino acids that differ between Zika virus strains H/PF/2013 and MR766. (D) All amino acids that differ between 111 Zika virus strains. Circles highlight the variability around the glycan loop in domain I. Sequences were obtained from the ViPR data bank (https://www.viprbrc.org/) on 23 September 2016 and analyzed with the Protein Variability Server (PVS) (166). Only Zika virus E sequences from complete genomes were used for the comparisons. Images were constructed by using data reported under PDB accession number 5IZ7 (64).

analyses have shown that the extent of cross-neutralization largely correlates with the amino acid sequence identity of E and led to the establishment of the dengue virus serocomplex (consisting of Den virus serotypes 1 to 4), the Japanese encephalitis virus serocomplex (also including West Nile virus), and the tick-borne encephalitis virus serocomplex (also including Powassan virus), among others (109). With respect to the E protein, yellow fever virus is almost as distantly related to the other mosquito-borne viruses as it is to the tick-borne viruses and most closely related to Wesselsbron virus, which is primarily an animal pathogen commonly found in sheep (110).

Zika virus has some intermediate position in this E protein tree. Together with its closest relative, Spondweni virus, which is endemic in the entire southwest of Africa, it is more closely related to the dengue virus than to the JE virus serocomplex or to YF and TBE viruses. Interestingly, a tree based on nonstructural protein 5 (NS5) (the viral polymerase encoded at the 3′ end of the viral open reading frame [ORF]) would place Zika virus closer to the JE virus serocomplex than to dengue viruses (66), suggesting an inhomogeneous evolutionary divergence of different parts of the flavivirus genome.

Overall, the picture of flavivirus serocomplexes (Fig. 3) indicates that cross-neutralization with polyclonal sera is usually lost when the amino acid sequence divergence of E is more than ~50%. An analysis of amino acids that are exposed at the surface of the E dimer and conserved among all mosquito-borne flaviviruses reveals that there is only a single notable small patch at the tip of DII, including residues of the FL (Fig. 4A), which in its occluded form does not seem to play an important role as an epitope in virus neutralization. Other assays, however, including hemagglutination inhibition (HI) assays and enzyme-linked immunosorbent assays (ELISAs) with E-containing antigens, are usually less specific than neutralization tests and reveal antigenic cross-reactivity among all flaviviruses (111). This assay-specific discrepancy can be explained by differences in the exposure of the FL under the conditions of such assays. In HI assays, it becomes exposed through an incubation step at acidic pH, which leads to E dimer dissociation and mimics the initial step of membrane fusion (Fig. 2B). In ELISAs, the FL, like other internal protein surfaces, can become more accessible through denaturation effects associated with protein adsorption onto solid phases (112).

It has to be kept in mind that the extent to which FL-specific and broadly cross-reactive antibodies contribute to virus neutralization can vary substantially between flaviviruses because of structural differences that control FL exposure in infectious

virions (e.g., prM cleavage and breathing) (see the corresponding sections above). Such antibodies seem to play a much more prominent role in the neutralization of dengue viruses than other flaviviruses, and this important aspect is discussed in more detail in Monoclonal Antibody-Defined Epitopes in Zika Virus E, below.

Zika Virus as a Single Serotype

Sequence analyses of old and new Zika virus strains have revealed three major genetic lineages (113): East African (including the first isolate from Uganda, MR766), West African, and Asian. The Asian lineage includes the first isolate from Asia (Malaysia, 1966; P6-740) and all contemporary strains from Asia, Oceania, and the Americas. The amino acid divergence among the E proteins of these strains ranges between ~6% between lineages and ~2% within lineages. Zika virus thus displays a low degree of E protein variation, not exceeding that found with other flavivirus species (e.g., TBE virus [~6%], YF virus [~5%], and WN virus [~7%]). This aspect is illustrated in Fig. 4C, which highlights surface-exposed amino acids that differ between a single pair of Zika virus strains, the prototypic French Polynesian strain (H/PF/2013) (GenBank accession number KJ776791) and the African strain MR766 (GenBank accession number KU720415). Figure 4D displays all positions of surface-exposed amino acids that are found to vary among the Zika virus strains available from the Virus Pathogen Resource data bank (ViPR) (https://www.viprbrc.org/). The variable patch highlighted by a circle corresponds to the site carrying the carbohydrate side chain. This loop is subject to deletions and mutations of as-yet-unknown significance (see "Structure of Zika Virus," above).

Given the prime role of E in virus neutralization, an important question is whether Zika virus can be regarded as a single serotype or whether the observed divergence of strains affects their neutralization by antibodies. Although the extent of variation is not unusually high, it is known from studies with other flaviviruses that even single-amino-acid exchanges can cause substantial differences in neutralization titers obtained with closely related variants, because they can modulate the formation of partially mature viruses and the extent of breathing (108).

This question was specifically addressed recently by Dowd et al., who analyzed the neutralization of six Zika virus strains by eight confirmed Zika virus convalescent-phase serum or plasma samples (114). Their analysis included two strains of the East African lineage (MR766, isolated in 1947 in Uganda, and ArB7701, isolated in the Central African Republic between 1976 and 1980) as well as four more contemporary strains from Brazil (2015), French Polynesia (2013), Thailand (2014), and the Philippines (2012). Neutralization assays were conducted with infectious viruses grown in mosquito and mammalian cells (to address possible cell-specific influences of the viral maturation state) as well as reporter virus particles. That study also included an analysis of mouse sera obtained after infection with African and Asian Zika virus strains. Overall, very little variation of mean neutralization titers was found, and it was therefore concluded that Zika virus is a single serotype (114).

Somewhat greater differences in cross-neutralization of African and American Zika virus strains were observed in recent work with postinfection sera of mice (115). Specifically, antibodies induced by the American strain (PRVABC59) (2015) neutralized the African strain (MR766) (1947) 10-fold less potently than the homologous American strain. These findings raised concerns in the context of cross-immunity and the possible spread of American Zika virus strains to Africa or Southeast Asia as well as the selection of strains for vaccine purposes. Interestingly, according to the data bank entries, the MR766 African strain (GenBank accession number LC002520) in this study lacked the E-glycosylation site (N_{154}DI), in contrast to the MR766 strain used in the study by Dowd et al., in which it was present (N_{154}DT) (GenBank accession number HQ234498) (114). This underlines the problem of possible strain variation due to *in vitro* passaging described in "Structure of Zika Virus," above, which may bias some of the *in vitro* neutralization results obtained with such strains. It therefore remains to be analyzed whether and to which extent the presence or absence of the single carbohydrate side chain in E can affect the results of neutralization assays and whether such differences

are relevant for naturally circulating strains. Concerning the selection of strains for vaccines, recent data from a rhesus macaque model are reassuring. Aliota et al. (116) demonstrated that animals infected with a prototypic African strain (MR766) were completely protected against challenge with a heterologous strain of the Asian genetic lineage. These data suggest that a single vaccine strain will protect against all naturally circulating Zika virus strains.

Serum Cross-Neutralization of Zika and Dengue Viruses

The intermediate position of Zika virus in the tree based on amino acid identities of E (Fig. 3) would be compatible with some degree of cross-neutralization between Zika and dengue viruses. This was indeed confirmed in several studies (albeit with limited numbers of samples) that analyzed Zika virus neutralization by dengue virus convalescent-phase serum or plasma samples (117–120).

The most comprehensive analysis so far was reported by Swanstrom et al. (119), who compared panels of convalescent-phase sera from cases of primary and secondary dengue virus infections for Zika and dengue virus neutralization. Only a few serum samples of both panels (17 and 16 samples, respectively) neutralized Zika virus, and the extent of cross-neutralization was low. However, there were a few notable exceptions of individual serum samples that contained moderate to high levels of Zika virus-neutralizing antibodies. Interestingly, the sera neutralizing Zika virus were not universally those with the highest dengue virus-neutralizing titers. A similar low and limited degree of cross-neutralization was also found by Dejnirattisai et al. (117), who showed that only 3 out of 16 dengue virus postinfection plasma samples could cross-neutralize Zika virus to some extent.

Even fewer studies are as yet available that have analyzed the reciprocal situation, i.e., cross-neutralization of dengue virus or other flaviviruses by Zika virus postinfection sera. Dowd et al. (114) showed that the neutralization titers of two Zika virus serum samples were much lower against dengue virus than against Zika virus and even lower against West Nile virus, which would be consistent with the position in the E protein divergence tree displayed in Fig. 3. Similarly, sera from Zika virus-infected mice cross-neutralized dengue 2 virus to only a limited extent (121).

The available data thus suggest that the conserved patches of surface-exposed amino acids shared between Zika and dengue virus E proteins (Fig. 4B) contain epitopes that can potentially induce strongly neutralizing antibodies albeit rarely. Studies with human monoclonal antibodies have indeed allowed the identification of such epitopes and the description of the structural basis for the link of cross-neutralization between Zika and dengue viruses (66). These epitopes together with Zika virus type-specific epitopes and broadly cross-reactive FL epitopes are discussed below with respect to their roles in Zika virus neutralization and enhancement of infection.

MONOCLONAL ANTIBODY-DEFINED EPITOPES IN ZIKA VIRUS E

Several panels of human and mouse MAbs have been used to describe the antigenic structure of Zika virus E at the single-epitope level. Some of these studies included the structural characterization of epitopes by X-ray crystallography of complexes between recombinant forms of E and antibody fragments in addition to analyses of neutralization and enhancement of infection. To avoid confusion in the description of these complex antibody panels, we describe three groups of MAb-defined epitopes separately according to their degrees of cross-reactivity in virus neutralization: (i) envelope dimer epitopes (EDEs) (responsible for Zika-dengue virus cross-neutralization), (ii) Zika virus type-specific epitopes, and (iii) broadly flavivirus-cross-reactive, FL-specific epitopes. Representative examples of these MAbs are summarized in Table 1. The data are derived from reports from different research groups (65, 66, 74, 122, 123), and a certain degree of variation of neutralization data from laboratory to laboratory has to be kept in mind in these comparisons. The aspect of antibody-mediated enhancement of infection and its relation to virus neutralization is discussed in Antibody-Dependent Enhancement of Zika Virus Infection, below.

TABLE 1 Zika virus-neutralizing activities of selected monoclonal antibodies with different degrees of flavivirus cross-reactivity

MAb	Epitope	IC_{50}[a]		Virus eliciting antibody	Source	Zika virus strain in NT[b]	Reference
		nM	ng/ml				
Zika-dengue virus cross-neutralization							
C8	EDE	0.095	14	Dengue virus	Human	H/PF 2013	66
C10	EDE	0.063	9	Dengue virus	Human	H/PF 2013	66
Zika virus type specific							
ZKA64	DIII	1.033	155	Zika virus	Human	H/PF 2013	122
ZKA190	DIII	0.08	12	Zika virus	Human	H/PF 2013	122
ZV-67	DIII	0.953	143	Zika virus	Mouse	H/PF 2013	74
ZKA185	Complex[c]	0.087	13	Zika virus	Human	H/PF 2013	122
ZKA230	Complex[c]	0.067	10	Zika virus	Human	H/PF 2013	122
ZIKV-117	Complex[d]	0.036	5.4	Zika virus	Human	H/PF 2013	123
Broadly flavivirus cross-reactive							
B12	FLE	>1,000	>150,000	Dengue virus	Human	H/PF 2013	66
2A10G6	FLE	1,667	250,000	Dengue virus	Mouse	SZ01	65

[a]IC_{50} values (50% inhibitory concentrations in a focus reduction neutralization test except for MAb 2A10G6, which was analyzed by a plaque reduction neutralization test) are derived from data given in the original publications in nanomolar concentrations, nanograms per milliliter, or micrograms per milliliter. For easier comparison, IC_{50} values are presented as both nanomolar concentrations and nanograms per milliliter.
[b]Strains H/PF 2013 and SZ01 have 99.8% identical amino acids in E. NT, neutralization test.
[c]Neutralizing non-E binding MAbs.
[d]"Dimer-dimer" interface DII.

Epitopes Providing a Link of Cross-Neutralization between Zika and Dengue Viruses

EDE stands for "envelope dimer epitope" (97) because these sites are dependent on the native dimeric assembly of E and comprise amino acids at the interface of both subunits. This group of complex quaternary epitopes was originally identified with human MAbs derived from dengue patients, and some of the corresponding antibodies were shown to cross-neutralize all four dengue virus serotypes at picomolar concentrations (97). The EDE MAbs have been subdivided into two groups (EDE1 and EDE2) on the basis of their sensitivity to the removal of the glycan linked to Asn153 in dengue virus E (Fig. 1F). Structurally, EDEs overlap the E-prM interaction site in immature virions (77), which can provide an explanation for their conservation across serotypes.

Some of these dengue virus-derived EDE MAbs (especially of the EDE1 group) were recently shown to cross-neutralize not only all four dengue virus serotypes but also Zika virus with similarly high potencies (66) (Table 1). X-ray crystallography of the Zika virus sE dimer in complex with EDE MAb fragments revealed structural details of this cross-neutralization. As displayed in Fig. 5A, the potently cross-neutralizing MAb EDE1 C8 binds to the upper surface of the Zika virus sE dimer, very similarly to its interaction with the dengue virus sE dimer (77). A comparison of the footprints of Zika virus and dengue 2 virus E dimers (Fig. 5B) revealed that the total surface area buried by this MAb is smaller in the Zika virus sE complex than in that of dengue 2 virus (900 $Å^2$ compared to 1,300 $Å^2$) (66). Most of this difference is accounted for by interactions with the dengue virus-specific glycan at Asn67, which is absent in Zika virus E (compare Fig. 1F and G). The main interactions, determining high-affinity binding and strong neutralization of EDE1 C8, are centered on β-strand *b* in DII and on conserved E-dimer-exposed elements of the fusion loop main chain, with additional contacts at the dimer interface (66). The epitope of another potently neutralizing EDE MAb (C10) (Table 1) was recently identified by high-resolution cryo-EM. Like C8, this antibody binds to the intradimer interface but also recognizes residues at the interdimer and interraft interfaces of the E herringbone (compare Fig. 1D and E) (124).

The EDEs are part of the conserved patches at the surface of E, as displayed in the comparison between Zika and dengue viruses (Fig. 4B). It is apparent that these surfaces have the potential to induce strongly neutralizing antibodies, but they are seemingly not immunodominant because Zika-dengue virus-cross-neutralizing antibodies are induced to a measurable extent in only a small proportion of dengue or Zika

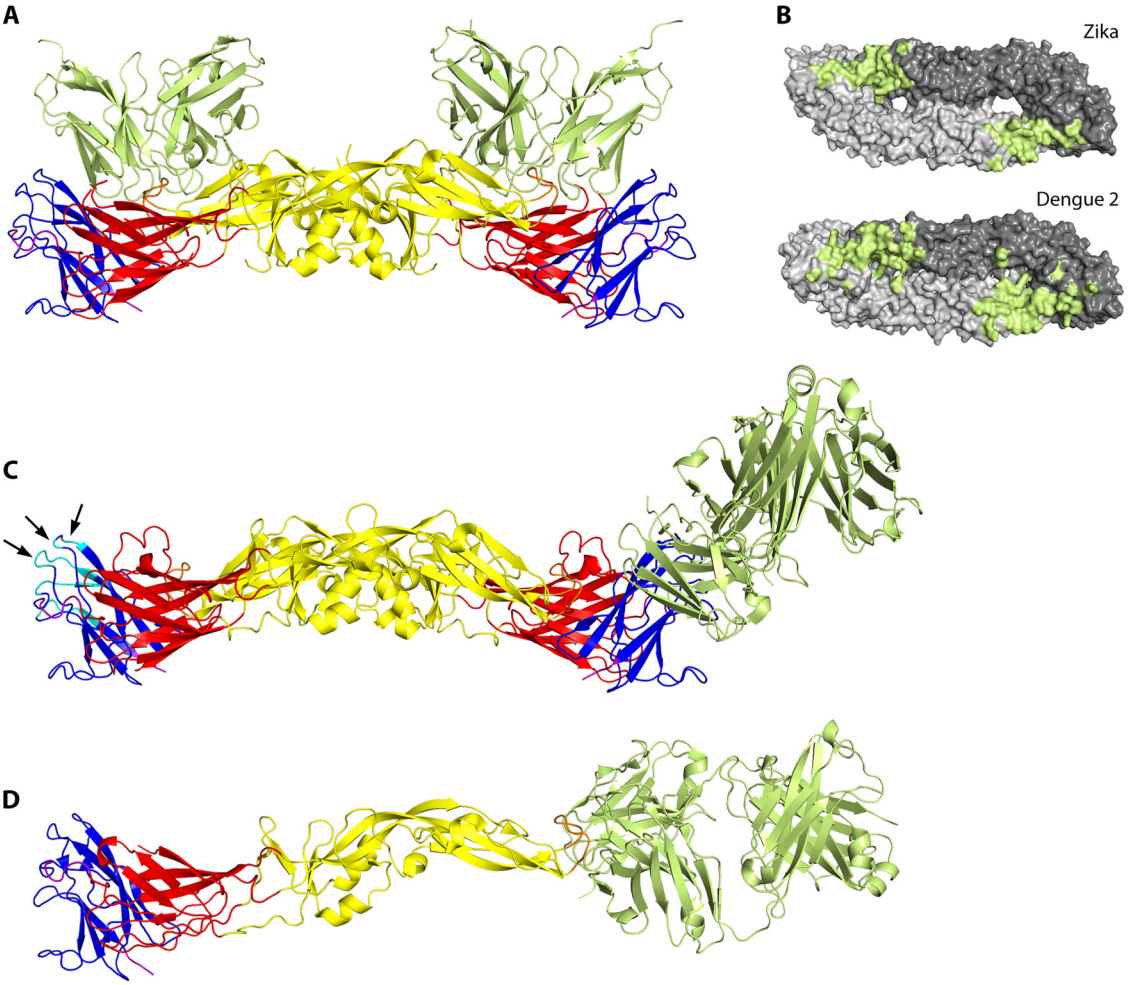

FIG 5 X-ray structures of Zika virus E dimers (A and C) and the E monomer (D) (side views) in complex with fragments of MAbs (green) (A, C, and D). (A) EDE-specific MAb C8 (PDB accession number 5LBS) (66). (B) Footprints of C8 in the Zika virus and dengue 2 virus E dimers in their top views (PDB accession number 5LCV [66] and PDB accession number 4UTC [77]). (C) DIII-specific MAb ZV67 (PDB accession number 5KVG [74] and PDB accession number 5LCV [66]). Only one Fab is shown on DIII on the right. In DIII of the second subunit (left), the loops forming the epitope (light blue) are highlighted by arrows. (D) FL-specific MAb 2A10G6 (PDB accession number 5JHL [65]).

virus-infected individuals. Irrespective of this aspect of immunodominance, the identification of EDEs provides a hint that the creation of vaccines protecting against both dengue and Zika viruses might be possible (66).

Zika Virus Type-Specific Epitopes

Potently neutralizing antibodies distinct from those recognizing Zika-dengue virus cross-reactive E dimer epitopes were recently identified in studies with MAbs obtained from Zika virus-infected humans (122, 123) and mice (74) (Table 1). These antibodies are type specific (at least they were shown to neutralize only Zika but not dengue viruses) and recognize two distinct classes of epitopes. Class 1 antibodies react with epitopes present on DIII only and are independent of the E dimer structure. They were described in both mouse and human MAb studies. The other class (so far described only in humans [122, 123]) apparently recognize complex quaternary epitopes that are Zika virus type specific and therefore distinct from the E dimer epitopes responsible for Zika-dengue virus cross-neutralization. The specific neutralizing activity of some type-specific antibodies of both classes is as high as that of the most potent EDE-specific antibodies (DIII-specific MAb ZKA190 and complex epitope-specific MAbs ZKA185 and ZKA230) (Table 1).

Detailed structural studies were performed with DIII-specific mouse MAbs that were shown to target three spatially distinct epitopes in DIII and to have variable neutralizing activity (74). The most potent MAbs (including ZV67) (Table 1) recognized the so-called lateral ridge epitope involving the A strand, the BC loop, the DE loop, and the FG loop (Fig. 5C), which is very similar to a previously characterized West Nile virus-specific mouse MAb (Fig. 5) (69, 125). An immunodominance of DIII was found in flavivirus-infected or immunized mice (83, 84) and appears to be a species-specific trait because no such dominance was found in humans (83, 85, 86, 101, 126) or horses (127).

No detailed structural information is as yet available for type-specific and potently neutralizing antibodies found in Zika virus-infected humans (Table 1), which recognize more complex structures than the DIII-specific MAbs, as described by Stettler et al. (122) and Sapparapu et al. (123). Antibodies such as ZKA185 and ZKA230 (Table 1) did not react with either recombinant DIII or recombinant sE and were identified only by functional screening in neutralization assays. Based on these properties, they were designated "neutralizing-non-E binding" (NNB) (122), which is probably somewhat misleading, because they certainly recognize E but presumably only in its complex quaternary herringbone-like arrangement or its specific curvature at the surface of mature virions. This also appears to be the case for MAb ZikV-117 (Table 1), for which mutational analyses suggest that it binds to DII across two adjacent dimers at the dimer-dimer interface (123). Epitopes of this complexity have already been structurally characterized for dengue and West Nile viruses by cryo-EM of virus-Fab complexes (75, 76, 78–80).

Broadly Flavivirus Cross-Reactive Epitopes

Antibodies directed to the highly conserved FL are responsible for the broad cross-reactivity observed between all flaviviruses in some assay formats (111, 128, 129), but the extent to which this structure elicits antibodies can vary considerably. In human dengue virus infections, FL antibodies appear to make up a major part of the total antibody response (100, 101, 130–132), whereas no such dominance was found in other flavivirus infections (85, 86). Broadly cross-reactive human MAbs were also isolated from Zika virus-infected patients (122), but structural details of their interaction with Zika virus E have not yet been resolved, and it remains to be shown at which proportions such antibodies are formed in the course of Zika virus infections.

Recently, the structure of a monomeric form of the Zika virus E protein with an FL-specific mouse MAb (Fab), originally produced against dengue 2 virus, was determined (65, 133) (Fig. 5D) and revealed an interaction that would be impossible with a native dimeric form of E. Nevertheless, the antibody (MAb 2A10G6) had Zika virus-neutralizing activity albeit with low potency (Table 1). Interestingly, in a comparative analysis of human MAbs of different epitope specificities, broadly flavivirus cross-reactive FL-specific antibodies (such as B12) (Table 1) were shown to neutralize dengue virus (although not as potently as the EDE MAbs) (Table 1) but failed to neutralize Zika virus despite high-affinity binding to the isolated Zika virus E protein (66). The explanation for this discrepancy between Zika and dengue viruses may be provided by differences in virus stability and the extent of breathing, which can result in differences in transient exposure times of the otherwise largely cryptic fusion loop (Fig. 1). Of note, FL epitopes may also be more accessible on the immature part of partially mature viruses, introducing a further variable to antigen presentation during flavivirus infections. Both the induction of a substantial proportion of FL antibodies in natural dengue virus infections and their higher neutralizing potency against dengue virus than against Zika virus as well as other flaviviruses suggest that dengue viruses are exceptionally prone to conformational dynamics and heterogeneities that expose the FL. This is supported by neutralization tests using the FL-specific MAb 2A10G6 (Table 1), which had moderate neutralizing activity against dengue viruses 1 to 4 (50% effective concentration [EC_{50}] values of 1,500 to 2,100 ng/ml) and 20-fold- to >250-fold-lower activity against West Nile, Japanese encephalitis, and tick-borne encephalitis viruses (133). Even with dengue viruses, the neutralizing potency of FL-specific antibodies

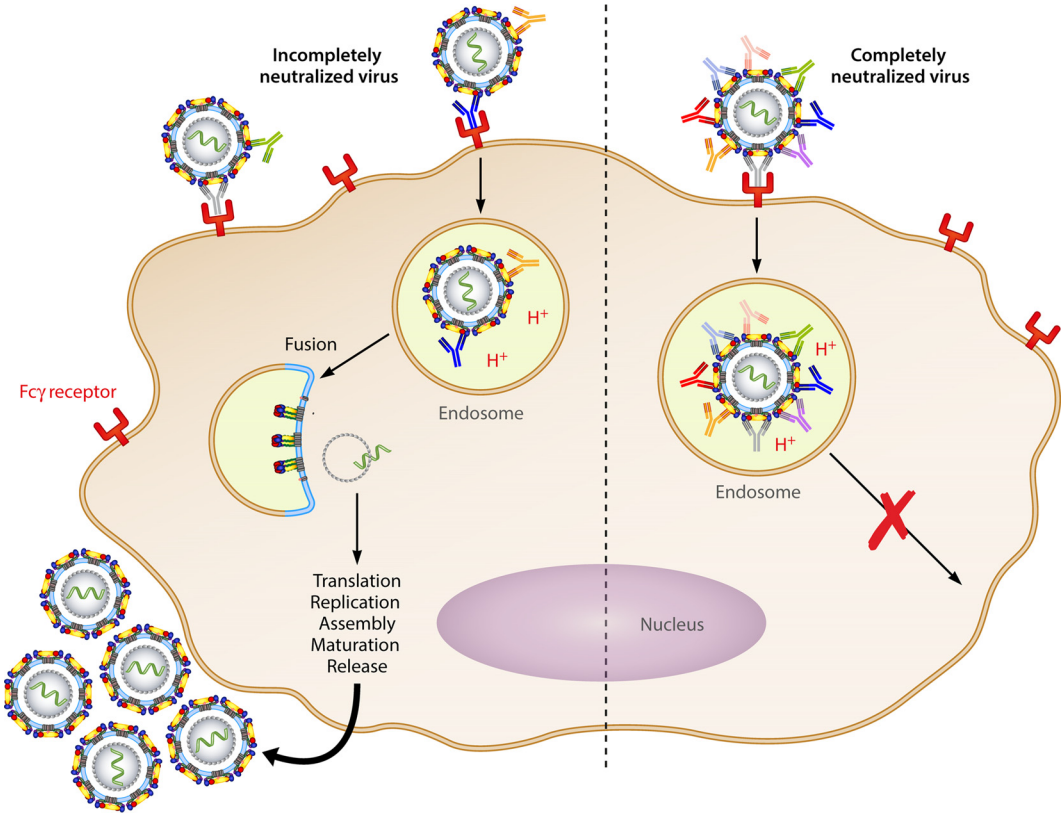

FIG 6 Schematic diagram of extrinsic antibody-dependent enhancement of infection in Fcγ receptor-positive cells. (Left) Virus-antibody complexes are internalized through Fcγ receptor-mediated endocytosis. Because of incomplete neutralization, the virus can fuse in the endosome and initiate virus production. (Right) Immune complexes containing completely neutralized virus can also be taken up through Fcγ receptor interactions but fail to fuse in the endosome and therefore do not lead to the production of progeny virus.

is rather low compared to potently neutralizing antibodies. Although Fcγ and complement-dependent effector functions can contribute to *in vivo* protection (134), the prospects for a universal flavivirus vaccine based on conserved FL epitopes appear to be rather poor. Antibodies to this site may even be detrimental because they have been shown to be potent mediators of antibody-dependent enhancement of infection.

ANTIBODY-DEPENDENT ENHANCEMENT OF ZIKA VIRUS INFECTION

As shown with many viruses of different virus families (reviewed in reference 28), antibodies at subneutralizing concentrations or insufficiently neutralizing antibodies can enhance virus production in myeloid cells such as monocytes, macrophages, and dendritic cells by mediating an increased uptake of infectious virus-antibody complexes through interactions with cellular Fcγ receptors. This phenomenon is now referred to as extrinsic antibody-dependent enhancement (ADE) of infection (Fig. 6) (135). Virus replication in such cells may be further increased by the suppression of innate antiviral cellular responses through antibody-dependent Fcγ receptor-mediated signaling, referred to as intrinsic ADE (135).

ADE has been implicated in the pathogenesis of severe forms of dengue (dengue hemorrhagic fever [DHF] and dengue shock syndrome [DSS]) in two situations: (i) as a consequence of the decline of maternal antibodies to subneutralizing concentrations in children born to dengue virus-immune mothers (136) and (ii) as a consequence of preexisting cross-reactive but insufficiently neutralizing antibodies in sequential infections with different dengue virus serotypes (136, 137). *In vitro*, flavivirus infection enhancement is usually measured by using permanent Fcγ receptor-positive cell lines derived from human lymphoma (U937) or leukemia (K562), which are barely infectible

in the absence of antibodies, in contrast to cells used for neutralization assays. In addition, animal models for demonstrating ADE and exacerbated disease *in vivo* have been established (138, 139). Based on evidence that ADE may be involved in the development of severe forms of dengue disease, this topic also raised immediate attention in the context of Zika pathogenesis, especially because dengue virus seroprevalence is >90% in some of the areas newly affected by Zika virus (140). It is still unclear whether preexisting dengue virus antibodies can contribute to fetal Zika virus infections or whether Zika virus antibodies may exacerbate dengue disease. However, a number of studies have addressed this important question in different experimental systems by using both polyclonal and monoclonal antibodies and demonstrated extensive mutual infection enhancement between dengue and Zika viruses.

Zika Virus ADE by Polyclonal Sera and E-Specific Monoclonal Antibodies

Already 30 years ago, when Zika was not yet considered a major public health problem, *in vitro* ADE of Zika virus infection was demonstrated with subneutralizing concentrations of homologous and heterologous immune mouse ascitic fluids raised against different flaviviruses (141). Similar results were recently obtained with human serum or plasma samples from dengue and Zika virus-infected individuals, which demonstrated a mutual enhancement of Zika and dengue virus infections *in vitro* (117, 118, 120).

Typically, sera that contained Zika virus-neutralizing antibodies did not enhance Zika virus infection at the highest serum concentrations tested but did so upon further dilution, leading to bell-shaped ADE curves using cells that are barely susceptible under standard infection conditions (117, 118, 120). The shift of peak enhancement to lower dilutions seemed to increase with the neutralizing activity of the serum sample, suggesting that the virus was completely neutralized at higher serum concentrations and unable to infect the cells (118) (Fig. 6). This indicates that the presence of potently neutralizing antibodies can prevent ADE, even when the virus is taken up as an immune complex by Fcγ receptor-mediated endocytosis. The extent of *in vitro* enhancement of Zika virus can vary strongly among individual serum samples and can even be completely negative (117).

Recent studies with large panels of Zika virus-reactive MAbs were especially revealing and provided important insights into the contribution of different antibody categories to ADE of infection (117, 118, 122). Overall, all MAbs, irrespective of their epitopes, were able to enhance infection over a broad but variable range of concentrations (117, 118, 122). These results are fully consistent with seminal studies conducted by Pierson and colleagues with West Nile virus, who showed that any antibody bound to the virus at an occupancy insufficient for virus neutralization can promote enhancement of infection in Fcγ receptor-positive cells (87). The ranges of concentrations leading to neutralization or enhancement, however, differed significantly between antibodies. Strongly neutralizing MAbs (e.g., EDE or DIII specific) (Table 1) (117, 118, 122) enhanced Zika virus infection only at relatively low concentrations but not at higher concentrations, consistent with a dominance of virus neutralization over enhancement in these cases. In contrast, antibodies with low neutralizing potency (directed to the FL [117]) (Table 1) displayed ADE even at high antibody concentrations.

An interesting exception to the ADE pattern seen with strongly neutralizing MAbs is revealed by a type-specific antibody recognizing a complex quaternary epitope at the surface of Zika virus (MAb ZKA230 [122]) (Table 1). Despite its strong neutralizing activity in susceptible cells, this antibody also mediated enhancement at high, and not only low, concentrations in K562 cells, probably because of differences in the mechanisms of neutralization of free viruses versus Fcγ receptor-internalized viruses (122). It can be hypothesized that such concentration effects are related to the capacity of the antibody to maintain or lose neutralization upon the Fcγ receptor-mediated uptake of the virus-antibody complex into cellular endosomes. Some antibodies are likely to dissociate from the virus under the acidic-pH conditions in this compartment, probably as a result of structural changes in E that are required for membrane fusion (Fig. 2). This

would be especially relevant for antibodies (like MAb ZKA230) (Table 1) that recognize the complex arrangement of E at the surface of infectious viruses, which is lost at acidic pH. Antibodies that potently neutralize the virus because they prevent virus attachment under normal conditions of cell entry (i.e., in the absence of Fcγ receptors) may therefore fail to neutralize the virus upon Fcγ receptor-mediated uptake under ADE conditions, because they cannot prevent membrane fusion in the endosome (Fig. 6).

The possible *in vivo* relevance of *in vitro* ADE was confirmed in a mouse model of dengue virus infection using AG129 mice, which lack the receptor for type I and II interferons (IFNs) (IFN-$\alpha/\beta/\gamma$ [122]). Preadministration of cross-reactive MAbs specific for E domains I/II (EDI/II) (raised from either Zika or dengue virus-infected humans) to such mice before infection with dengue 2 virus caused severe symptoms and death, in contrast to control mice, all of which survived under the conditions of infection, proving the potential of Zika virus-reactive antibodies to increase dengue virus pathogenicity *in vivo*. The authors of that study also attempted to measure enhanced disease after Zika virus infection, but the mouse model used was not equally suited and did not yield conclusive results (122).

Importantly, the Zika virus infection-enhancing activity of poorly neutralizing MAbs and polyclonal serum/plasma can be effectively counteracted and even abolished by the presence of strongly neutralizing antibodies but may be dependent on the structural nature of the epitopes recognized by such antibodies. In a homologous setting of Zika virus ADE by Zika virus immune plasma, Stettler et al. (122) demonstrated that enhancement of infection could be completely blocked by a strongly neutralizing DIII-specific MAb but only partially by a broadly cross-reactive EDI/II MAb. Similarly, Dejnirattisai et al. (117) found that MAbs to the structurally different ED epitope potently inhibited the Zika virus-enhancing activity of dengue virus plasma, whereas no such effect was seen with FL-specific MAbs.

Both of these studies indicate that the presence of sufficient concentrations of potently neutralizing antibodies can protect against infection-enhancing effects of poorly neutralizing antibodies in Fcγ receptor-positive cells. Such a dominance of neutralizing over enhancing antibodies is consistent with the mechanism of extrinsic ADE shown in Fig. 6, provided that the antibody interaction is not weakened or lost in the endosome, e.g., as a consequence of structural rearrangements of the viral envelope at acidic pH (see above).

These findings point to the potential impact of the individual variability of antibody compositions present at the time of secondary flavivirus infections. The relative proportions and specificities of antibody subsets produced in the course of flavivirus infections or vaccinations can vary considerably among different flaviviruses and among individuals infected with the same flavivirus or immunized with flavivirus vaccines (83, 85, 86, 142). This individual heterogeneity of antibody specificities in polyclonal responses may therefore be a key parameter that determines beneficial, neutral, or detrimental influences on infections with heterologous flaviviruses.

Role of Anti-prM Antibodies in ADE

The formation of partially mature but infectious virus particles is a characteristic feature of flavivirus maturation (see "Partially Mature Virions," above). The extent to which these particles are formed in infected hosts is currently not known, but, at least in dengue virus infections, humans produce a substantial proportion of antibodies specific for prM (100, 131). These antibodies are nonneutralizing or barely neutralizing *in vitro* (100, 131) and also nonprotective in animal models of West Nile virus infection (143). However, they are cross-reactive among dengue virus serotypes and display strong infection-enhancing activity *in vitro* and *in vivo* (100, 144, 145). It was therefore proposed that they may play a key role in the immunopathogenesis of dengue virus by contributing to ADE in the course of secondary dengue virus infections (145).

The situation may be different with other flaviviruses and strongly dependent on the circulation of immature or partially mature virions in infected humans. Relatively low antibody responses to prM were found in postinfection sera of TBE virus and after live

YF virus vaccination (85, 86). The possible role of prM and prM antibodies in the biology of human Zika virus infections remains to be investigated. Considering its presumed impact on dengue virus pathogenesis, this question should have a high priority in forthcoming Zika virus research.

IMMUNOPROPHYLAXIS OF ZIKA

The newly emergent problem of Zika virus in the Americas and especially its association with congenital malformations have prompted developments in several areas of potential intervention, including vector control, therapeutics, and, of course, immunoprophylaxis (146). Although the list of exploratory projects is quite long (146), there are still only a few publications on Zika virus vaccines as well as on antibodies for passive immunotherapy. Both approaches have the major goal of preventing prenatal Zika virus infection and associated clinical consequences such as microcephaly, other nervous system malformations, and pregnancy-related complications, as defined in the WHO Zika Strategic Response Plan (147).

Zika Vaccines

The long-standing success of effective flavivirus vaccines, specifically those against YF, JE, and TBE viruses (148–150), and the recent licensure of a vaccine against dengue virus (151) suggest that the development of a Zika virus vaccine is technologically feasible. The WHO continually monitors the progress of Zika virus vaccine candidates and has established a vaccine pipeline tracker website (152), which includes more than 30 nonclinical and clinical candidates under active development by academic groups and/or industry (as of 29 November 2016). The approaches pursued include purified inactivated virus, live recombinant virus, subunit vaccines, virus-like particles, live vectored vaccines, and nucleic acid-based vaccines. The programs are at different stages of development, and two phase 1 clinical studies in humans started in 2016 (153). According to the WHO (http://www.who.int/immunization/research/meetings_workshops/WHO_Zika_vaccine_TPP.pdf?ua=1), immunization of women of childbearing age is considered to be of the highest priority as a strategy to prevent congenital infections in the current situation. As a longer-term goal, vaccination of the general population from childhood to adulthood may be an effective means of reducing virus circulation in regions of endemicity by inducing population immunity (154).

The first reported preclinical data with experimental Zika virus vaccines are encouraging (155, 156). In a mouse model of Zika virus-induced viremia, complete protection, even after a single vaccination, was conferred by a conventional inactivated whole-virus vaccine adjuvanted with aluminum hydroxide as well as a DNA vaccine expressing the full-length M and E proteins of Zika virus (155). Accidentally, this construct was designated "prM-E," but according to the given sequence information, it contained only M and therefore should read "M-E" (153). Other DNA constructs used in this study, encoding full-length E alone or truncated forms of E, were only partially protective and reduced viremia to various degrees (155). E-specific antibody titers were the key correlates of immune protection, as demonstrated in challenge experiments after passive immunization with postvaccination sera. These vaccination trials were extended to nonhuman primates (rhesus monkeys), which were immunized with the inactivated virus vaccine, the M-E DNA vaccine, or a recombinant rhesus adenovirus serotype 52 vector vaccine also expressing M and E (156). Although all three vaccines conferred complete protection against contemporary Zika virus strains from Brazil and Puerto Rico after two immunizations, the inactivated-virus vaccine induced substantially higher neutralizing antibody titers (more than 10-fold) than the DNA or adenovirus vector vaccines.

New and promising DNA vaccine candidates that express full-length prM and E (Zika virus strain form French Polynesia) and lead to the formation of subviral particles (SVPs) similar to those produced as natural by-products of virus replication were recently described (Fig. 2) (157). SVPs carry the E protein in a conformation corresponding to that on whole virions (158), and recombinant forms thereof have been shown to be

excellent flavivirus immunogens (reviewed in reference 159). DNA vaccines optimized for SVP production induced high titers of neutralizing antibodies in mice as well as nonhuman primates (rhesus monkeys) and mediated protection (prevention of viremia) upon virus challenge in monkeys (157). Importantly, protection was correlated with serum neutralizing activity, and these studies may lead to the definition of a protective threshold of vaccine-induced neutralizing antibody titers (157). Taken together, existing animal protection data and the observation that Zika virus can be considered a single serotype (see "Zika Virus as a Single Serotype," above) give cause for optimism that vaccines for preventing Zika virus infections and hopefully vertical transmission can become available in the not-too-distant future.

New structural information on epitopes recognized by the most potently neutralizing antibodies (see Monoclonal Antibody-Defined Epitopes in Zika Virus E, above) may provide leads to even more innovative approaches in future vaccine design, such as the development of epitope-focused immunogens. Domain III of E was identified as a target of some of the most potent Zika virus-neutralizing antibodies (Table 1), and corresponding structural analyses provided a hierarchy of neutralization efficacy among epitopes located in DIII (74). The authors of that study therefore propose engineering of variants of DIII that target the antibody response toward the most effective regions in this domain, thus improving its immunogenicity compared to previous attempts using DIII as a flavivirus vaccine (160).

Another potential novel vaccine strategy may make use of the ED epitopes for designing artificial immunogens that render these epitopes immunodominant. EDEs represent a link of efficient cross-neutralization between Zika and all four dengue viruses (66, 117), and the elucidation of their atomic structure may provide a lead toward the rational structure-based design (161) of a universal dengue-Zika virus vaccine. Given the complex quaternary structure of these epitopes, such an approach certainly constitutes a formidable scientific challenge.

It is difficult to predict how much time will be needed to obtain licensure of a Zika virus vaccine for human use (154). Safety issues such as the induction of nonprotective but potentially infection-enhancing cross-reactive and/or prM-specific antibodies relative to neutralizing antibodies will certainly need to be addressed in future trials. The association of Zika virus infections with increased numbers of GBS cases will also require specific attention when assessing outcomes of vaccination trials (154). The fact that women of childbearing age (including pregnant women) are primary target groups for Zika virus vaccines may represent an obstacle to the use of live vaccines or vaccines based on new technologies that have not yet been approved for use in humans. Such vaccines, however, may offer advantages for routine immunization programs for the general population (154).

Antibodies and Passive Immunization

Another option to prevent congenital Zika virus infections in pregnant women would be the administration of human or humanized antibodies (74, 119, 122, 123). Passive immunoglobulin therapies have been established previously for preventing birth defects caused by other viruses such as rubella virus, varicella-zoster virus, or cytomegaloviruses (162–164). The demonstration that some of the Zika virus E-specific antibodies have very strong neutralizing activities (Table 1) makes them attractive for passive immunotherapy. Indeed, several studies in experimental mouse models have shown that symptomatic Zika virus infections could be effectively prevented by passive immunization. This was demonstrated with antibodies specific for DIII (74, 122), complex whole-virion-dependent epitopes (122, 123), an ED epitope (119), and even an FL epitope albeit at a very high antibody concentration (65). The antibodies used in these studies and their specific neutralizing activities are highlighted in Table 1 (C10, ZKA64, ZV-67, ZIKV-117, and 2A10G6). Whether passive immunization can protect pregnant women and prevent congenital infections remains an open question. As indicated by Zhao et al. (74), the pregnant mouse models developed so far appear not to be suited for conclusive experiments because of differences in placental anatomy compared to

that of humans. Experiments in nonhuman primates may therefore be necessary to address this important question before application of passive immunotherapy in humans (74).

A matter of concern is possible enhancement effects due to the enhancement of infection mediated by passively administered antibodies, which could predispose to more severe outcomes of Zika virus infection when antibody levels reach subneutralizing concentrations (see Antibody-Dependent Enhancement of Zika Virus Infection, above). In the case of cross-reactive antibodies, such effects may also aggravate heterologous infections with dengue viruses, as shown in an animal disease model (122). Fortunately, a solution to this problem is provided by the introduction of mutations in the Fc part of the antibodies that abolish their interaction with Fcγ receptors and, as a consequence, their potential of inducing ADE, without impairing their neutralizing activity. Such mutant antibodies (with replacements of L223A and L235A and therefore designated LALA mutants) were shown to be as effective as their wild-type counterparts in preventing Zika virus infection in animal models (122). Importantly, the administration of a DIII-specific LALA MAb also completely blocked enhanced Zika virus infection caused by antibodies in sera from dengue virus-immune individuals (122). Passive immunotherapy with potently Zika virus-neutralizing LALA mutant antibodies may thus also serve to prevent the enhancement of infection due to preexisting cross-reactive, nonprotective antibodies.

CONCLUSIONS AND PERSPECTIVES

The sudden expansion of Zika virus from Asia to Oceania and the Americas as well as its possibly changed pathogenicity were unexpected and a reminder of the potential threat imposed by obscure arboviruses slumbering in tropical forests. The risk of explosive outbreaks is highest when these viruses, like Zika virus, are transmitted by anthropophilic vectors such as *Aedes aegypti* mosquitoes and can use humans as a principal vertebrate host. Vector control therefore appears to be the most important and immediately beneficial measure of disease prevention in this case (147).

Studies related to the antigenic structure of Zika virus, the major topic of this review, have yielded good and bad news at the same time. The good news is that epitopes and corresponding monoclonal antibodies that can provide potent virus neutralization *in vitro* and protection in animal models have been identified. Such antibodies may be developed into therapies for humans in critical situations to prevent neonatal infection and associated disease (74, 119, 122, 123). Vaccine development is also very promising, and a plethora of conventional and innovative approaches are being pursued in this area of research (146). Considering the success of existing flavivirus vaccines (148–150) and based on data reported so far (155–157), there should be no principal technical impediments to the induction of protective immunity against Zika virus by vaccination. For the more distant future, studies of structures recognized by potently neutralizing MAbs can even provide clues for new vaccine technologies, including the design of epitope-focused vaccines (74) and universal dengue-Zika virus vaccines (66).

The bad news is that existing experimental studies do not rule out the possibility that immunopathological mechanisms mediated by nonprotective antibodies may contribute to neonatal infections by Zika virus, similarly to their described role in severe manifestations of dengue virus infections (27, 153). With Zika virus, antibody-dependent enhancement of infection has so far been demonstrated only *in vitro* and was shown to be especially strong with cross-reactive antibodies (recognizing the fusion loop) derived from previous dengue virus infections. In dengue virus, there is a second group of antibodies, directed to another envelope protein (prM) found in partially mature virions, which are strong mediators of infection enhancement. The potential contribution of this class of antibodies to enhanced infection by Zika virus will certainly be the target of future studies.

Zika virus infection enhancement by dengue virus antibodies is a matter of concern because Zika virus spreads in regions where dengue virus is hyperendemic, and most of the population must be considered to have potentially enhancing antibodies. The

problem is even more complex since antibodies induced by Zika virus were shown to enhance dengue virus infections not only *in vitro* but also in a mouse model (122), raising the possibility of reciprocal disease aggravation with this pair of viruses. Conclusive answers as to the role in human disease will require epidemiological studies as well as an animal model that is sufficiently similar to humans (possibly nonhuman primates) and allows the systematic measurement of antibody-mediated effects on disease outcome. Preliminary data obtained with a low number of rhesus macaques ($n = 4$) preinfected with dengue viruses followed by infection with Zika virus did not reveal evidence of increased peak viremia compared to dengue-naive controls (165).

The rapid progress made in Zika virus research gives cause for optimism that measures of immunoprophylaxis and/or immunotherapy will become available in the not-too-distant future. A major focus will be on the avoidance of antibody-mediated immunopathological consequences of such interventions. In the case of passive immunization, this problem appears to be solved through the development of mutant antibodies that are incapable of mediating ADE of infection. Understanding whether infection-enhancing antibodies are induced by vaccination and how potential unwanted effects can be avoided will remain topics of future vaccine research. The challenge of product development and the work of licensing authorities is increased by the fact that women of childbearing age and/or pregnant women are a primary target population for the application of antibodies and vaccines.

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