ABSTRACT Dengue provides the most abundant example in human medicine and the greatest human illness burden caused by the phenomenon of intrinsic antibody-dependent infection enhancement (iADE). In this immunopathological phenomenon infection of monocytes or macrophages using infectious immune complexes suppresses innate antiviral systems, permitting logarithmic intracellular growth of dengue virus. The four dengue viruses evolved from a common ancestor yet retain similar ecology and pathogenicity, but although infection with one virus provides short-term cross-protection against infection with a different type, millions of secondary dengue infections occur worldwide each year. When individuals are infected in the virtual absence of cross-protective dengue antibodies, the dengue vascular permeability syndrome (DVPS) may ensue. This occurs in around 2 to 4% of second heterotypic dengue infections. A complete understanding of the biologic mechanism of iADE, dengue biology, and the mechanism of host responses to dengue infection should lead to a comprehensive and complete understanding of the pathogenesis of DVPS. A crucial emphasis must be placed on understanding ADE. Clinical and epidemiological observations of DVPS define the research questions and provide research parameters.

This article will review knowledge related to dengue ADE and point to areas where there has been little research progress. These observations relate to the two stages of dengue illnesses: afferent phenomena are those that promote the success of the microorganism to infect and survive; efferent phenomena are those mounted by the host to inhibit infection and replication and to eliminate the infectious agent and infected tissues. Data will be discussed as “knowns” and “unknowns.”

Antibody-dependent enhancement (ADE) is a phenomenon involving infectious IgG antibody immune complexes that mediate the worsening of diseases involving a wide spectrum of microbes and vertebrates. ADE is a new type of Gell-Coombs immunopathology: type I, IgE-mediated immediate hypersensitivity; type II, antibody-mediated acute immune complex disease; type III, IgG-mediated complement-dependent foreign antigen immune complex disease; type IV, cell-mediated immune and autoimmune diseases; and type V, IgG immune complex enhancement of microbial infection in Fc-receptor (FcR)-bearing cells. Three of these immunopathologies are mediated by IgG antibodies. Type V immunopathology differs in function from type II and III immunopathologies in that immune complexes are not directly cytotoxic but serve to increase disease severity by regulating the productivity of intracellular microbial infection. In type II immunopathologies, IgG antibodies are often directed at autoantigens and include acute rheumatic fever where microbial antigens mimic antigens in various human tissues, generating an immune response that breaks down immune tolerance. In type III immunopathologies foreign antigen-antibody complexes are often trapped in the basement membranes of endothelial linings. Examples include acute serum sickness.
glomerular nephritis, and postimmunization diseases such as breakthrough measles and respiratory syncytial virus infections in vaccine recipients that result in destructive complement-fixing virus-IgG immune complexes predominantly in the lung (1).

An early report of the ADE phenomenon, in vitro, was by Hawkes, who observed a greater number of plaques in chick embryo fibroblast monolayers infected with Murray Valley encephalitis virus (MVEV) preincubated with high dilutions of chicken antisera than in virus-only controls (2). In further studies the authors concluded that this phenomenon was the result of antibodies stabilizing the spontaneous degradation of MVEV (3). A different explanation emerged when enhanced infection of dengue virus (DENV) was observed in cultures of peripheral blood mononuclear cells from dengue-immune compared with nonimmune subhuman primates (4). This phenomenon was subsequently attributed to enhanced growth of DENV infection in primary monocytes and macrophages in the presence of nonneutralizing enhancing dengue antibodies (5–7). It was shown subsequently that MVEV infection enhancement occurred in functional chicken macrophages that comprise 2% of chick embryo fibroblast monolayers (8). Because of the conformational requirement that Fcγ receptors and IgG Fcγ termini be of the same vertebrate phylogenetic class, ADE in chick embryo fibroblasts was observed only when MVEV antibodies were raised in chickens, not in mammals (2). Because monocytes and macrophages were identified as the principal hosts of in vivo DENV infection, the phenomenon of ADE was suggested as an immunopathologic mechanism (10–16).

Different lines of scientific inquiry over the past four decades have sharpened our understanding of microbial antibody-mediated pathogenesis mechanisms in vertebrate hosts. During initial studies of ADE it was assumed that the observed increased growth of virus, which in some cases was 100- to 1,000-fold, was the result of phenomena extrinsic to mononuclear phagocytes such as an increase in rates of attachment or internalization of immune complexes to target cells resulting in an increased number of infected cells compared with controls (6, 7, 17). These mechanisms were studied in mouse macrophage-like cells when West Nile immune complexes attached to FcR-bearing cell surfaces more rapidly compared with naked virus particles (18, 19). With feline infectious peritonitis virus an increased number of peritoneal macrophages were infected in vitro in the presence compared with the absence of antibodies (20). It is also possible that immune complexes were internalized more rapidly than was naked virus as observed in an HIV-1 model (21).

These mechanical explanations of ADE changed radically when human macrophages were infected in vitro using Ross River virus (RRV) immune complexes, or in mouse models in vivo. In humans, acute infections with RRV often evolve to a postinfection arthritis of many months’ duration. It was observed that arthritis patients’ synovial cells stained for RRV antigens and synovial fluids contained interferon-γ (IFNγ). In an attempt to model this phenomenon, chronic RRV infections were established in mouse macrophage cell lines and in primary human monocytes/macrophages (22). Unexpectedly, the incubation of RRV with diluted RRV antiserum resulted in enhanced infection in these cells through a complex intracellular process involving the suppression of innate cellular immunity by immune complexes. This involved a reduction of the production of reactive nitrogen radicals via NOS2 and the downregulation of INF-α and IFN-β production by abolishing IRF-1 and nuclear factor-KB gene expression. Also, there was a marked increase in IL-10 gene transcription and protein production (23, 24). This immune complex suppressive phenomenon required an infectious agent since the ligation of FcγR by zymosan-antibody complexes in the presence of RRV did not ablate antiviral transcription (24). Thus, rather than simply involving an increase in the number of infected cells, ADE in RRV is a complex intracellular phenomenon involving increased intracellular production of virus as a result of immune complex suppression of innate cellular immunity.

These observations were quickly expanded to dengue. The DENVs are a group of four closely related members of the Flavivirus genus. DENV-1 through -4 share 60 to 70% genetic homology and are inoculated by the bite of infected Aedes aegypti. Initial infections with any of the four DENVs raise protective type-specific antibodies, but the dominant population of antibodies are cross-reactive and nonneutralizing. These efficiently enhance infection by a different DENV type (5, 25–29). In vitro studies of FcR-bearing cells show that monoclonal dengue antibodies of many specificities may form infectious immune complexes, the major requirement being attachment to a virion surface antigen at a subneutralizing antibody concentration (30, 31). In practice, antibodies directed at surface epitopes that are not involved in virus entry efficiently produce ADE (32). Indeed, the high rate at which infants acquire severe dengue disease during their first dengue infection when maternal polyclonal dengue antibodies have degraded below protective levels is a unique illustration of the ADE phenomenon in human medicine (10, 33–39). Studies of the pathogenesis of innate and acquired host immune
responses to many acute and chronic human and animal infectious diseases show evidence that cross-linking of IgG immune complexes with Fcγ receptors increases intracellular infection, thus contributing to disease severity by a mechanism labeled intrinsic ADE-iADE (40, 41). iADE distinguishes intracellular mechanisms from the extrinsic mechanisms of ADE—an increase in infectivity, infection rate, or the number of infected cells by immune complexes compared with infection with the microorganism only. Extrinsic and intrinsic ADE have been measured as contributing a 3-fold or a 100-fold increase in virus production, respectively (42).

The ADE phenomenon has attracted wide interest in viral pathogenesis research because many viruses replicate in macrophages in vivo, and this phenomenon is correlated with enhanced disease in many partially immune vertebrate hosts (43–45). Here, the focus will be on iADE in dengue infections as the mechanism that controls the conversion of a mild self-limited acute illness to the dengue vascular permeability syndrome (DVPS). DVPS is the underlying pathophysiological mechanism of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) as defined by the 1997 WHO Technical Guidelines (Dengue Haemorrhagic Fever: Diagnosis, Prevention, Treatment and Control. 1997. World Health Organization, Geneva, Switzerland, p. 1–84).

ADE as a pathogenesis mechanism should lead ultimately to a coherent explanation for all the phenomena that comprise DVPS. The basic physiological disturbances and the critical timing of DVPS during the course of a dengue illness are described in Table 1. DVPS usually presents in individuals immune to a single DENV who experience a heterotypic dengue infection (46). A small fraction of cases occurs during a second heterotypic (third) DENV infection (47). DVPS also occurs during primary dengue infections in individuals circulating passively acquired dengue antibodies. In animal models the simple passive transfer of dengue antibodies sensitizes hosts to DVPS during a subsequent DENV infection. Based on these observations, it is conceivable that DVPS might occur during dengue infections in individuals who had previously received blood transfusions from dengue-immune donors. This phenomenon has not yet been reported. But whatever the case, DVPS regularly occurs during primary dengue infections in infants born to women who previously had acquired multiple dengue infections (33, 34, 48). While in dengue-endemic countries this phenomenon contributes 5% of all hospitalized cases, in general, it is poorly studied and vastly under-reported.

As in all infectious diseases, dengue infections progress through phases during which afferent and efferent phenomena predominate. Afferent phenomena are those that improve or potentiate the infection process and pathogenicity of the infecting microorganism. Efferent phenomena are those that counter the pathogenic potential of the infecting microorganism and that lead to the elimination of the infection. For most infections, researchers studying afferent phenomena focus on microbial offensive and defensive weapons, but in dengue, enhancing antibodies and the specific immune complexes that are made contribute powerfully to potentiate infection and disease outcome in dengue. It is my contention that DVPS is the result either of pathogenic factors released directly by infected tissues and/or as an outcome of normal host efferent efforts to control and end infection. In either of these cases, disease severity is directly related to the mass of dengue-infected tissues. Because DVPS in individuals experiencing second dengue infections is essentially identical to that occurring in infants, it is crucial that pathogenesis hypotheses and research efforts focus on unitary mechanisms of DVPS that explain passively as well as actively acquired dengue immunity. The known and unknown afferent and efferent factors contributing to ADE regulation of dengue disease severity will be identified and discussed below according to the outline in Table 2.

### TABLE 1 Dengue vascular permeability syndrome

<table>
<thead>
<tr>
<th>A Dengue syndrome that occurs late in the course (on or near defervescence) of an acute dengue illness consisting of:</th>
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<tbody>
<tr>
<td>(a) <strong>Thrombocytopenia</strong></td>
</tr>
<tr>
<td>(b) <strong>Altered hemostasis:</strong> most commonly prolonged bleeding time and/or elevated aPTT (activated partial thromboplastin time) and/or elevated prothrombin time</td>
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<tr>
<td>(c) <strong>Activated complement,</strong> by classical and alternative pathways</td>
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<tr>
<td>(d) <strong>Elevated liver transaminase enzymes</strong></td>
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<tr>
<td>(e) <strong>Vascular permeability:</strong> Clinically significant loss of fluid and small macromolecules (e.g., albumin) into interstitial spaces, most commonly to serosal cavities</td>
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### KNOWN S

**Afferent**

**Host genetic factors contributing to susceptibility to DVPS**

Many human genetic factors have been identified as being significantly associated with increased or decreased incidence of DVPS during secondary dengue infections. These have been described in recent reviews (49). These factors will not be further considered or discussed here.
TABLE 2 Context for understanding “known” and studying “unknown” factors that contribute to the pathogenesis of the dengue vascular permeability syndrome via ADE

<table>
<thead>
<tr>
<th>Knowns</th>
<th>Afferent:</th>
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<tbody>
<tr>
<td>a. Host genetic factors enhancing susceptibility to DVPS</td>
<td>b. “Enhancing” antibodies</td>
</tr>
<tr>
<td>i. Sensitizing infections</td>
<td>ii. Attributes of iADE</td>
</tr>
<tr>
<td>iii. Role of infection sequence</td>
<td>iv. Role of different myeloid cells</td>
</tr>
<tr>
<td>v. Role of different FcγRs in iADE</td>
<td>c. Role of DENV in ADE</td>
</tr>
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<td>iii. DENV-2 genetic differences associated with rapid enhancement of the severity of DENV-2 infection 20 years after infection with DENV-1</td>
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**Enhancing antibodies**

**The sensitizing infection**

Human experimentation has established that there is a DENV-2 infection refractory period of three months following an initial DENV-1 infection (50). Possibly related to this protective phenomenon, sera obtained shortly after a primary DENV infection were found to have abundant heterotypic antibodies that formed large immune aggregates on the surface of primary human macrophages that were neutralized in solution but not eliminated via phagocytic clearance (51). After this refractory period, second dengue infections occur, and a portion are expressed as clinical illness. Varying amounts of heterotypic neutralizing antibodies are raised following a primary dengue infection. The natural histories of antibody responses following infection by DENV-1, -2, -3, or -4 including heterotypic antibodies are often unknown.

Infection-enhancing antibodies are an observed risk factor for enhanced dengue disease (52), and an enhanced peak viremia (measured before antibody response starts) has been shown to be an anticipatory correlate of severe
Dengue Antibody-Dependent Enhancement: Knowns and Unknowns

disease (53, 54). Recent pathology studies of human autopsies and tissues from mouse models have firmly established the central role of monocytes, macrophages, and immature and mature dendritic cells as infected target cells (13–16, 55).

In dengue-endemic countries monotypic infections with DENV-1, -2, -3, or -4 sensitize individuals to disease accompanying a first heterotypic (second) dengue infection. This phenomenon accounts for around 95% of hospitalized and carefully defined DVPS (47). The clinical outcomes of first heterotypic dengue infection are time dependent. First heterotypic infections occurring at less than a two-year interval are partially protected, resulting in inapparent infections or mild disease (56–59). After that time, DVPS has been observed in 2 to 4% of all second dengue infections combined (for a discussion of the pathogenicity of different sequences of heterotypic dengue infection, see below) (30). The frequency and severity of DVPS increases as the interval between the first and second infections lengthens. This phenomenon was observed when hospitalization and case fatality rates were compared among individuals who were 15 to 39 years old when they experienced first heterotypic DENV-2 infections either 4 or 20 years after a DENV-1 infection.

In 1977 to 1979, around 45% of the population of Cuba (at that time, age groups <1 to 40 were dengue naïve) was infected with DENV-1, a virus introduced into Cuba only once. DENV-2 circulated on the island in 1981, 4 years after DENV-1, and DENV-2, at an interval of 20 years. Hospitalization rates per 10,000 hospitalizations were 8.2 times higher than at those at a 4-year interval (60). The case fatality rate was 4.7 times higher when second heterotypic DENV-2 infections occurred at a 20-year interval compared with a 4-year interval (60). This observation was subsequently correlated with a sustained decline in heterotypic DENV-2 neutralizing antibody titers over a period of 20 years following DENV-1 infections in 1977 to 1979 (61). Heterotypic antibodies have been observed to be important in the modulation of second DENV infections in cultures of primary human monocytes (52). Low dilutions of preinfection sera from children who had inapparent secondary DENV-2 infections almost invariably reduced or neutralized DENV-2 infections in primary human monocytes, while preinfection sera from children who developed an illness requiring hospitalization had little or no detectable heterotypic neutralizing antibodies (52). This observation suggests that low levels of heterotypic neutralizing antibodies (most were anti-DENV-1) did not prevent DENV-2 infection but downregulated DENV-2 clinical responses. In this study, around one-fifth of monotypically dengue-immune school children lacked heterotypic DENV-2 antibodies measured in primary human monocyte cultures and developed DHF when infected by DENV-2. This is virtually the same ratio as for the occurrence of shock during sequential DENV-1 then DENV-2 in Rayong, Thailand (62). Prior infection with two different DENVs (second heterotypic dengue infection) also sensitizes to DVPS, but at a frequency about 50-fold lower than a single DENV infection (47).

In the in vitro ADE literature antibodies to many flaviviruses enhance DENV infection in FcR-bearing cells (63). Should this be true for in vivo infections, opportunities abound for flavivirus group virus infections to sensitize to enhanced dengue infections. In Southeast Asia the DENVs cocirculate with Japanese encephalitis (JE), in India with JE and West Nile, in Pakistan with West Nile, in Australia with the Kunjin strain of West Nile, and in most of the American region with yellow fever or yellow fever 17D vaccine. Importantly, in Thailand it was observed that children with previous wild-type JE infections or who were immunized with killed JE vaccine developed mild overt dengue illness at a higher frequency than did flavivirus-susceptible patients infected with the same DENV (64). Dengue infections in JE-immune patients not only increased the rate of mild overt dengue disease, but the ensuing illness lasted 2 times longer than dengue illnesses in nonimmune patients. This establishes the ability of JE antibodies to mildly enhance human dengue infections. The in vitro correlates of this observation have not been described. Prior to this observation, based upon data trends in a large vaccine study, it was thought that JE vaccination reduced the severity of DHF accompanying a second dengue infection (65).

A large amount of literature has emerged on the role of nonneutralizing antibodies in enhancing DENV infections. It has been observed that a considerable fraction of all antibodies circulating after a first heterotypic dengue infection are directed at prM (32, 66). prM antibodies target immature or partially immature DENV via the pr peptide. A significant fraction of all DENVs produced in vitro are immature or partially immature, as are an unknown fraction of DENVs circulating during natural human infections (67). Immature DENVs cannot be processed in the endosome to result in infection. prM antibodies were found to be highly cross-reactive with all DENV serotypes and generally to have poor neutralizing capacity (32, 68). Recent studies showed that prM antibodies enhance the infectivity of noninfectious
immature DENV particles (32, 69, 70). Monoclonal DENV antibodies directed at several E domains have been found to interact with immature particles, resulting in infection enhancement in FcR-bearing cells (71, 72).

The attributes of iADE

This is a remarkably complex phenomenon, triggered by attachment of IgG-microbial pathogen complexes to FcRs of many classes, resulting in messages being passed and expressed via the cytoplasmic tail. Much of what we know about FcR signaling comes from other pathogens—Leishmania amastigotes, for instance. The importance of macrophage receptors in the generation of cytokines in Leishmania-infected macrophages was recognized when interleukin (IL)-12 production in BALB/c mouse bone marrow macrophages in response to lipopolysaccharide was suppressed after ligation of FcR, complement, or scavenger receptors (73). Both mRNA synthesis and protein secretion were diminished to near undetectable levels following receptor ligation. Suppression was specific to IL-12 since TNFα production was not inhibited. Also, the ligation of mouse FcγR with immune complexes was shown to enhance the production of IL-10 (74). Stimulation of mouse bone marrow macrophages by lipopolysaccharide resulted in some IL-10 production, but the addition of red blood cell (RBC) opsonized with IgG antibodies dramatically enhanced IL-10 production. Immune complexes not only induce activated macrophages to produce IL-10, but they also induce both macrophages and dendritic cells to switch off their production of IL-12 (73, 75). The IL-10 induction by IgG-amaystigotes did not occur in macrophages from mice lacking the common gamma chain that signals through FcγR 1, 3, and 4, indicating that one or all of these three receptors were involved. Subsequent studies using defined immune complexes demonstrated that all three of the FcγRs that signal through gamma were capable of signaling for IL-10 production in macrophages (76). The implication from these studies is that in some settings, IgG itself biases the immune response toward a Th2-type response. Indeed, for some species of Leishmania, chronicity of infection requires that amastigotes be coated with IgG (77). This phenomenon is now very well established (78–80).

That IL-10 induction by ligation of FcγR was a generic process was demonstrated with a nonmicrobial antigen (81). Lipopolysaccharide-treated BALB/c mouse macrophages when exposed to ovalbumin alone developed T cell responses driven to Th-1 and characterized by the production of IFNγ. When the same antigen was complexed with IgG anti-ovalbumin, T cell responses were driven to Th2 and produced IL-4. This Th2-like phenotype was stable and was retained when the T cells were subsequently restimulated under nonbiasing conditions. Mice vaccinated with IgG-opsonized ovalbumin made high levels of IgG Ab of the IgG1 isotype. The T cell biasing and its reversal via FcγR ligation was also observed in vivo. Using macrophages from gene knockout mice, the production of IFNγ and IL-4 by T cells was shown to be controlled by the macrophage cytokines IL-12 and IL-10, respectively. These and other studies demonstrate that the ligation of FcγR on activated macrophages reverses the Th1 biasing that accompanies innate immune responses to microbial products.

How do antibody-coated amastigotes result in the production of IL-10 by macrophages? Ligation of macrophage FcγR produces a rapid and enhanced activation of two mitogen-activated protein kinases: ERK and p38 (82). The activation of ERK leads to the phosphorylation of serine 10 on histone H3 at the il-10 gene, making the promoter more accessible to transcription factors generated in response to p38 activation (83). Activation of both mitogen-activated protein kinases was required for IL-10 synthesis. In addition to ERK activation, an inflammatory stimulus, such as low-molecular-weight hyaluronic acid from the extracellular matrix, must also be present. The combination of these two signals resulted in the superinduction of IL-10 (84). Macrophages lacking FcγR, or macrophages treated with an inhibitor of spleen tyrosine kinase that is activated following FcγR ligation, failed to activate ERK and consequently failed to produce IL-10 following infection with Leishmania amastigotes.

During in vitro ADE DENV infection in the THP-1 cell model (human monocyteic Fcγ receptor-bearing continuous cell line) intracellular DENV production was increased as a result of idiosyncratic Fcγ-receptor signaling (85). When immune complexes ligate FcγRI and FcγRIIA, at least two of the following types of suppression pathways are expressed: DAK, Atg5-Atg12, SARM, and TANK plus the positive Th2 cytokine regulator, IL10. Collectively, these phenomena downregulate antiviral responses in ADE-infected target cells. DAK and Atg5-Atg12 of RIG-I/MDA5 abolish expression of RIG-I/MDA5 and weaken the RIG-I/MDA5 signaling pathway as monitored through levels of downstream signaling molecules: IPS-1, IKKi, TRAF-3, TBK-I, etc. One outcome is decreased production of type-I IFN as well as IFN-activated antiviral molecules (86). Activation of SARM and TANK results in expression blockage of Toll-like receptors (TLR) 3, 4, and 7 (87). This inhibits MyD88-dependent and MyD88-independent signaling
pathways, resulting in another route for type I IFN suppression. As a result of at least these two suppression pathways, ADE-infected THP-1 cells secreted reduced levels of type I IFN and at the same time suppressed the transcription and translation of IL-12, IFN-γ, and TNF-α, facilitating the expression and synthesis of the anti-inflammatory cytokines. ADE infection also suppressed the innate anti-DENV mediator, nitric oxide radicals, by disrupting the transcription of the inducible nitric oxide synthase (iNOS) gene transcription factor, IRF-1 (85). This suppressive mode is believed to be mediated by IL-10 activity. IL-10 is synthesized at an early phase of ADE infection in THP-1 cells. In this experimental setting, IL-10 not only induces Th2 biasing but operates via the suppressors of cytokine signalling (SOCS) system to suppress the JAK/STAT signaling pathway, resulting in suppression of iNOS gene expression and reduction of nitric oxide radical production. The viral enhancement effect of IL-10 is abolished with small interfering RNA specific to the IL-10 gene (86). It can be concluded that in vitro, iADE infection not only facilitates viral entry but also modifies innate and adaptive intracellular antiviral mechanisms, resulting in enhanced DENV replication.

Critically, the same responses are observed in vivo. Genome-wide transcriptomes from peripheral blood mononuclear cells collected during the acute phase from children with dengue fever (DF) or DHF were compared using microarray analysis (88). Patients with DHF had decreased levels of NO, reduced IFN transcript in peripheral blood mononuclear cells, and increased IL-10 blood levels compared with patients with milder illness. IFN gene upregulation and IFN-α production were significantly elevated in patients with mild compared with severe dengue illness. In other studies, during the acute stage of severe disease increased production of IL-10 and downregulation of multiple IFN regulatory genes were noted (89–91). The protective role of IFN in moderating dengue infection has been demonstrated in a mouse model and suggested for humans with DF (92–94). The precise role of immune-complex-elicited IL-10 production on the clinical evolution of severe dengue infections is not well understood but may be responsible for the observed Th1 to Th2 shift in DHF (95).

Pathologic studies of human tissues have established monocytes, macrophages, and immature and mature dendritic cells as significant targets for DENV infection (13–16, 96, 97). In humans, secondary dengue infections follow a stereotypical course with severe outcomes, shock or gastrointestinal hemorrhage, accompanying vascular collapse that results from capillary permeability occurring around the time of defervescence (98). Indirect evidence suggests that cytokines mediate dengue vascular permeability. Much work has been directed at the measurement of cytokine blood levels in patients late in the acute phase, just prior to onset of shock (99, 100). High levels of viremia early in the disease and high levels of pro-inflammatory and immunomodulatory cytokines including IL-10 late in the disease are associated with severe outcomes (101).

However, during ADE-infection of primary monocytes, IL-10 synthesis peaked at the same serum dilution that produced peak virus yield (42). In addition, point mutations at the IL-10 promoter, positions -1082 A/G, -819 C/T, and -592 C/A, result in polymorphism that differentiates monocytes into high, intermediate, and low IL-10 producers. How these phenotypes correlate with disease severity requires more investigation.

**Role of infection sequence**

Second DENV infections can occur in 12 combinations, at least 10 of which have been documented to result in hospitalized disease (47). The sequence of infection may be highly determinative of disease severity. Secondary DENV-2 infections resulted in shock syndrome patients, while secondary DENV-1, -3, and -4 did not in the 1980 cohort study in Rayong, Thailand (62). The specific infection sequences associated with DSS cases were known from virus isolations in acute phase sera and antibodies in pre-illness sera or by applying the original antigenic sin phenomenon to paired sera (102). Although secondary DENV-1 infections were most common that year, DSS occurred only during secondary DENV-2 infections. Burmese workers came to a similar conclusion in their 1984 to 1988 longitudinal seroepidemiological study in Yangon, Myanmar (103). By contrast, in an Indonesian study DSS was associated with sequences ending in DENV-1, -3, and -4, but not DENV-2, even though secondary DENV-2 infections occurred (104). DENV-3 was associated with an outbreak of DHF/DSS in Tahiti in a population that had prior infection experience with DENV-1 and DENV-2 (105). Also in Tahiti, DENV-1 circulating in 2001 was enhanced to produce severe clinical disease by antibodies to DENV-2 that had circulated 4 to 5 years earlier (106).

**Role of different myeloid cells**

Much early published work on dengue and Ross River virus (RRV) used primary human mononuclear phagocytes to study iADE. To date, virtually all research has been carried out using DENV-2. When dengue iADE was tested in four different primary human myeloid
cells derived from the same peripheral blood leukocyte (PBL) donors, viral infection and cytokine responses differed significantly (42). Human monocytes, activated macrophages, and mature dendritic cells supported ADE, while immature dendritic cells did not. Infection of macrophages by DENV-2 alone or as fully neutralized immune complexes stimulated high levels of α and β IFN, and these were downmodulated under ADE conditions and replaced by secretion of IL-6 and TNFα. Type I IFNs were not produced or suppressed by iADE infection of monocytes with DENV-2 (42, 107). However, during ADE infection of primary monocytes, IL-10 synthesis peaked at the same serum dilution that produced peak virus yield (42). In addition, point mutations at the IL-10 promoter, positions -1082 A/G, -819 C/T, and -592 C/A, result in polymorphisms that differentiate monocytes into high, intermediate, and low IL-10 producers. It should be noted that FcR-bearing continuous cell lines that are incapable of producing interferon have been used to detect extrinsic ADE but do not detect or produce iADE. A prime example is K562 cells, widely used to measure enhancing properties of dengue antibodies (108). Published research results using K-562 cells are often at odds with results obtained using primary human myeloid cells—the one group measuring extrinsic ADE and the other iADE (52, 109).

Role of different FcγRs in iADE
When the ability of DENV immune complexes to be internalized following interactions with human FcγRIIA and FcγRIIIA was studied in a model system, the FcγRIIA mediated both iADE and immune phagocytosis, while FcγRII mediated uptake of immune complexes via phagocytosis (110). Genes for human FcγRIIA have been transfected into continuous monkey kidney cells, transforming them into cells capable of detecting and expressing the capacity of DENV immune complexes to be neutralized or to enhance infections. These systems, employing FcγRIIA have been used to assay in vivo viremia and to measure the protective versus enhancing properties of sera containing mixtures of DENV and antibodies (111–115).

In the Leishmania mouse model, it was observed that mouse IgG1 and IgG2a/c induce IL-10 from mouse macrophages in vitro equally well but through different FcγRs: IgG1 through FcγRII, and IgG2a/c primarily through FcγRII but also through FcγRIII. In sharp contrast, mice lacking IgG1 develop earlier and stronger IgG2a/c, IgG3, and IgM responses to Leishmania mexicana infection and yet are more resistant to the infection (116). Thus, IgG1, but not IgG2a/c or IgG3, is pathogenic in vivo, in agreement with prior studies indicating that FcγRIII is required for chronic disease. This calls into question the assumption that mouse macrophages, which should secrete IL-10 in response to both IgG1 and IgG2a/c immune complexes, are the most important source of IL-10 generated by IgG-FcγR engagement in L. mexicana infection.

Role of DENVs
Enhanced growth of DENV-2 in primary human monocytes
A single study suggests that DENVs isolated from patients with differing degrees of disease severity may themselves be biologically different. The biological behavior of wild-type DENV-2 isolated from children with mild secondary dengue infections was compared with DENV-2s from children with grade I-III dengue hemorrhagic fever. All isolates were made in C6-36 cells from children presenting to Children’s Hospital during the 1980 DENV-2 outbreak in Bangkok, Thailand (117). DENV-2 that had been passaged in C6-36 cells only once or twice from mild illnesses replicated to lower titers in cultures of human primary monocytes either with or without enhancing concentrations of polyclonal dengue antibodies than did DENV-2 strains isolated from DHF patients. The small number of subjects with mild illness jeopardizes the statistical significance of these observations. Nonetheless, these preliminary observations suggest the possibility that viral factors, whether surface antigens, attachment sites for entry into leukocytes, or intrinsic replication properties in human mononuclear phagocytes, might contribute to enhanced DENV infection and to the severity of the disease.

Differences in ability of DENV-2 strains to be neutralized by DENV-1 antibodies
Differences in genetic and viral structure among DENV-2 viruses that are associated with disease outcome during a first heterotypic dengue infection are well established, and the impact of these differences has been well studied (118). The actual sites on the virion that mediate these differences in human disease expression are still unknown. The first DENV to be recovered in the northern hemisphere was DENV-2 TR 1751 (119). Dengue outbreaks prior to World War II have been attributed to DENV-2 by serological studies in Panama and Cuba (120, 121). In 1963, DENV-3 was introduced into the northern hemisphere, first recognized in Puerto Rico (122). The geographic extent and intensity of transmission of these viruses was never measured, but conditions
Dengue Antibody-Dependent Enhancement: Knowns and Unknowns

DENV-2 genetic differences associated with rapid enhancement of the severity of DENV-2 infection 20 years after infection with DENV-1

A dramatic increase, month to month, in case fatality rates and the severity of dengue disease was observed in humans of all ages who, in 1981 and 1997, experienced a DENV-2 infection 4 and 20 years after a DENV-1 infection (129). During a 2001 to 2002 Havana outbreak a similar rapid increase in disease pathogenicity was observed in individuals who experienced a DENV-3 infection 24 years after infection with DENV-1 (130–132). Initially, it was hypothesized that this phenomenon might be caused by the emergence of neutralization escape mutants, as DENV-2 was serially passaged in individuals circulating DENV-1 antibodies (129). As discussed above, DENV-1 infections produce heterotypic antibodies that partially neutralize DENV-2. These might be expected to generate neutralization escape mutants that are no longer neutralized and that could produce enhanced infections and disease. It was suggested that the proportion of individuals infected with escape mutants might increase with time, producing the observed month-to-month increases in pathogenicity.

In January 1997, the sensitive Cuban surveillance system detected dengue cases in Santiago de Cuba just two months after illness in the index case (identified retrospectively). Acute phase sera were sent to the Havana laboratory for serological testing and virus isolation. The DENV-2 strains that circulated in 1981 and 1997 in Cuba both belonged to the American/Asian genotype circulating in the Americas since 1981. Twenty-nine DENV-2 isolates were obtained during the early (low pathogenicity) and the late (high pathogenicity) stages of the 1997 epidemic. The 1997 Cuban DENV-2 strain amino acid alignment showed a substitution methionine/threonine at position E340 specific for viruses isolated from Cuba, Venezuela, and Martinique (133). This nonconserved substitution is located in an antigenic region containing multiple T- and B-cell epitopes. Another nonconservative change leucine/glutamine at E131 was observed in Jamaica/83 and Cuba/97 isolates. Remarkably, only two nonconserved substitutions in the E gene were found between the Jamaica 1983 and the 1997 Cuban strains, indicating that there has been very little in situ evolution of DENV-2 following its introduction from Asia. Of interest, the Cuban DENV-2 isolates maintain the presence of N at position 390 of Asian DENV-2 strains, a site predicted to be a determinant of the American genotype DENV-2 that causes only mild disease (126). As the E gene sequences from these isolates were found to be conserved over the period of the outbreak, the
hypothesis that envelope gene escape mutants contributed to the observed increased disease severity is negated (133).

The complete genes of six of the 1997 DENV-2 isolates were sequenced. A strong conservation of structural genes and proteins and of the noncoding regions was again noted. However, nucleotide substitutions were observed in NS genes, notably in NS1 and NS5 (134). Most synonymous mutations generally correlated with the time of sampling. It was possible to divide the isolates into two groups based upon five substitutions—those from the beginning of the 1997 epidemic (January to February) and from the latter parts of the epidemic (June to July). One of these five nucleotide substitutions produced a functionally significant amino acid replacement, threonine (thr) to serine (ser), at residue 164 in the NS1 protein. An alignment of this NS1 protein sequence in GenBank revealed that amino acids in this region are highly conserved. DENV-2 strains usually have Thr at this position, with the exception of the Thailand strain PUO-280 of the Asian I genotype, which has Ser, like the latest Cuban isolates (135). Strains of DENV-2 may differ significantly in the structure of genomic viral RNA 3′ nontranslated region (NTR), and these structural differences have also been correlated with mild disease (126). However, the DENV strains from the 1997 epidemic had no difference in their 3′ NTR or 5′ NTR compared with other Asian/American strains.

In further work, nucleotides 379 to 601 were sequenced from 15 viruses from different periods of the outbreak. Data from six DENV-2 strains sequenced previously were included (133). Two additional complete DENV-2 strains were sequenced directly from serum samples (130). Of 23 DENV-2 strains obtained during the course of the epidemic, five viruses from the first part of the epidemic had thr at position 164, while the isolates on and after 25 February had ser at this position (130, 136). By July, mosquito control efforts were well established and cases began to decline, with a marked reduction in cases in August. The switch from threonine to serine at NS1 position 164 appears to have been fixed within a few months of the virus having been introduced into Cuba.

Genetic differences in DENV-2 isolates recovered from disease of enhanced severity 4 years after DENV-1 and 8 years after DENV-3

Similar dengue disease phenomena were observed among pediatric patients in Managua, Nicaragua, and adults in Taiwan (137, 138). Further information has been published (136). In Nicaragua, a prospective seroepidemiological cohort study made possible a complex dissection of the contributing host and viral factors involved (138). In the dengue season of 2005–2006, 34 patients with DENV-2 infections were admitted to Hospital Infantil Manuel de Jesus Rivera in Managua, 10 with the diagnosis of DHF/DSS (30%). During 2006–2007 and 2008–2009, the severity of DENV-2 disease increased dramatically (85% of cases were secondary infections). Of 102 children with DENV-2 infections admitted to the hospital during these nonconsecutive transmission seasons 64% had DHF/DSS, an unparalleled increase in this hospital’s 20-year experience with dengue admissions. Differing from the situation in Cuba, where DENV-2 introductions in 1981 and 1997 were rapidly controlled by effective vector control, dengue transmission in Nicaragua was continuous.

In Cuba, introductions of DENV-2 in 1981 and 1997 and DENV-3 in 2001 were preceded by DENV-1 infections in 1977-1978, whereas in Nicaragua DENV-3 had circulated in Managua in 1994 to 1998, DENV-2 had circulated in 1999 to 2002, and DENV-1 in 2002 to 2005, with DENV-2 again in 2005 to 2009. During this latter period a large number of DENV-2 strains were collected from children with dengue diseases of all degrees of severity. Over 200 of these viruses were subjected to full-length or partial sequencing, and as had been observed in Cuba, a stable clade shift was noted: NI-1 to NI-2B in 2008–2009. This replacement event was associated with nine nonsynonymous amino acid mutations (R97K in capsid [C], K94R in nonstructural protein 1 [NS1], and P245T in nonstructural protein 3 [NS3], N245S in NS4B, M492V in envelope [E], L279F in NS1, and K200Q, T290I, and R401K in nonstructural protein 5 [NS5]) and four mutations in the viral NTR. Clade NI-2B peak viremia titers were higher than viremias with clade NI-1 viruses in children with the diagnosis of dengue fever. By analyzing the age distribution of cases, it was concluded that NI-1 DENV-2 produced disease with an apparent increase in pathogenicity in children who were immune to DENV-1 because there was cross-protection when infections occurred at short intervals, while more severe disease was observed at longer intervals. By contrast, clade NI-2B DENV-2 infections produced disease of increased pathogenicity in children of all ages who had been infected by DENV-3 during 1994 to 1998. The authors noted that clade NI-2B DENV-2 strains grew to higher titers than clade NI-1 viruses in C6/36, primary human dendritic cells, U937 DC-SIGN, and K-562 cells, but ADE was not studied using DENV-3 antibodies. This group made no attempt to grow clade NI-1 or NI-2B DENV-2 strains in the presence of human DENV-1 or DENV-3 antibodies.
in vitro in primary human monocytes or macrophages to model ADE infections.

**Efferent**

The incubation period of disease ending in DVPS is unknown but is presumed to be the same as that of dengue fever. In children, progression of the illness is characteristic. A relatively mild first phase with an abrupt onset of fever, malaise, vomiting, headache, anorexia, and cough may be followed after 2 to 5 days by rapid deterioration and physical collapse (139). In Thailand, the median day of admission to the hospital after the onset of fever is day 4. In this second phase, the patient usually has cold and clammy extremities, a warm trunk, a flushed face, and diaphoresis. Patients are restless and irritable and complain of midepigastric pain. Frequently, scattered petechiae appear on the forehead and extremities, spontaneous ecchymoses may develop, and easy bruising and bleeding at sites of venipuncture are common findings. Circumoral and peripheral cyanosis may occur. Respirations are rapid and often labored. The pulse is weak, rapid, and thready, and the heart sounds are faint. The pulse pressure frequently is narrow (<20 mm Hg); systolic and diastolic pressure may be low or unobtainable. The liver may become palpable two or three finger breadths below the costal margin and usually is firm and nontender. Early sonograms show thickening of the gallbladder duct and after initiation of intravenous resuscitation may show thickening of the gall bladder duct, perivesicular edema, ascites, and pleural effusions. At this same time chest radiographs may show unilateral (right) or bilateral pleural effusions. Approximately 2 to 5% of patients have gross ecchymosis or gastrointestinal bleeding. After a 24- or 36-hour period of crisis, convalescence is fairly rapid in children who recover. The temperature may return to normal before or during the stage of shock.

There is evidence of an important human dengue resistance gene. Epidemiologic studies of the 1981 Cuban outbreak demonstrated a higher risk for DHF/DSS in white than in black individuals (121). A search for DHF/DSS in black children in Haiti revealed no cases, despite the presence of high dengue type 1, 2, and 4 infection rates and circulation of the Southeast Asian genotype dengue 2 viruses (140). Several HLA antigens have shown differing frequencies in DHF/DSS cases and controls (141). Early in the acute vascular permeability stage of secondary DENV infection, rapid activation of the complement system occurs (142, 143). During shock, blood levels of C1q, C3, C4, C5, C6, C7, C8, and C3 proactivator are depressed, and C3 catabolic rates are elevated. The blood clotting and fibrinolytic systems are activated (139, 144). As yet, neither the mediator of vascular permeability nor the complete mechanism of altered hemostasis has been identified unequivocally. The kinin system apparently is not involved. Studies show that levels of tumor necrosis factor, interleukin-2, and interferon-γ are elevated at the time of vascular permeability (144). The strongest correlates to vascular permeability are inverse levels of platelet counts and reductions in accelerated partial thromboplastin time (145). Capillary damage allows fluid, electrolytes, protein, and, in some instances, red blood cells to leak into intravascular spaces. This internal redistribution of fluid, together with deficits caused by fasting, thirsting, and vomiting, results in hemoconcentration, hypovolemia, increased cardiac work, tissue hypoxia, metabolic acidosis, and hyponatremia. A mild degree of disseminated intravascular coagulation, plus liver damage and thrombocytopenia, could contribute additively to produce hemorrhage.

In serial blood samples taken early in the illness of individuals experiencing secondary dengue infections it is possible to identify peak viremia or dengue NS1 concentrations that can be measured. These were used successfully to observe enhanced dengue infections and to predict subsequent disease severity (53, 54, 146). It is critical that viremia be measured in the absence of antibodies—a very difficult task.

Because of the delay of onset of serious vascular permeability to around defervescence, there is a widespread hypothesis that vascular permeability is somehow related to T cell activity—specifically, the result of dengue-infected cells being attacked by activated T lymphocytes. Cytokine production should be quantitatively related to the number of infected target cells. The reduced risk for DHF/DSS in protein-calorie malnourished children and the increased risk for DHF/DSS in girls versus boys are consistent with the hypothesis that a competent immune elimination system is available to generate the cytokines that produce DHF/DSS (147–149).

It is widely held that the process of eliminating DENV-infected cells generates a cascade of chemokines and cytokines that contribute to the pathophysiology of dengue disease syndromes. This has been termed “a perfect cytokine storm” (150). Given the evidence of increased infected cell mass in severe dengue infections, the cytokines generated by interactions between virus-infected cells and the host immune response would appear to be quantitatively proportional to viral load and not exaggerated or “abnormal.” Since dengue is not a cytophilic virus, cellular infection proceeds until the infected cell is eliminated. A wide misconception is that peak viremia that occurs early in infection represents...
peak “viral load.” In fact, the quantity of virus in the blood during the course of infection only describes the kinetics of extracellular virus clearance. As discussed above, it is likely that elimination of DENV-infected cells continues well after onset of antibody production, resulting in peak cellular infection (viral load) at around the time of defervescence, and cellular infection is not eliminated until well after the end of viremia. Of interest, a major effort failed to detect circulating CD8+ T cells during the late acute illness phase of DHF patients (151). This suggested to the authors that CD8+ T cells might be located only in the tissues where DENV replication had occurred. Alternatively, as in a Japanese encephalitis model, the antibody component of the adaptive immune response may play a much more important role in terminating dengue infections than previously thought (152).

UNKNOWNs
The elements of the preceding section are briefly reprised here. Ideally, these are accompanied by the identification of research questions and provision of detailed research protocols. The state of dengue research on the intracellular ADE is very primitive. The best I can do is to suggest a research approach. In almost all instances the approach involves the direct investigation of primary human myeloid cells with infectious DENV immune complexes. It is crucial that researchers use cells from donors. Long ago, it was shown that dengue-immune plasma can only be approached by investigating primary human myeloid cells.

Afferent
Host genetic factors enhancing susceptibility to DVPS
Human genetic factors promoting dengue disease severity are not discussed in this article. The contribution of human genetic variables will be better understood when they can be placed into a rational understanding of how extrinsic phenomena and normal human biology control ADE.

“Enhancing” antibodies
Sensitizing infections
As described above, the initial single dengue infection is known as the “sensitizing infection.” The immune response to this infection participates in ADE accompanying infection with a heterotypic dengue virus.

• Studies of iADE have almost exclusively focused on DENV-2. A high priority is to extend studies as quickly as possible to the complete range of DENV. ADE should be tested using type-specific polyclonal human antibodies and not mouse or even human monoclonal antibodies. ADE is complex. The test systems should retain essential complexity.

• DVPS in infants occurs under completely different immunological circumstances than during first heterotypic dengue infections. The failure of anyone in the past four decades to seriously study this very important and biologically unique phenomenon is simply mind-boggling. I am sorry to say I do not regard the published studies of Libraty and Simmons as having asked serious research questions (35, 36). Kliks led the way (34). Why not use maternal serum as a surrogate of cord blood for each individual infant and test it along with the virus isolated? This unique clinical phenomenon not only illustrates the role played by dengue antibodies in modulating disease expression but, also, the bifurcated role of dengue antibodies—protection for several months after birth at high concentrations and enhancing infections some months later at subneutralizing concentrations.

• The contribution of immature DENV particles to iADE is of interest. Immature DENV virions are not infectious for human myeloid cells, but in the presence of enhancing dengue antibodies, ADE infection occurs readily (32, 69). It has been surmised that immature DENVs are released into circulation during human infections, as antibodies to prM (immature DENV antigen) frequently are observed (32).

Attributes of iADE
The precise mechanisms fueling enhanced production of DENV in monocytes and macrophages require further study, as neither the production of IL-10 nor suppression of type I interferon are critical to the iADE phenomenon.

Role of infection sequence
There are at least 12 sequences of infection that make 12 unique immune complexes. We already have preliminary data that suggest that the outcome of infection differs according to the specific immune complexes (e.g., JE antibody-DENV immune complexes). One of the most critical absent studies is the characterization
of infection, cytokine, and innate immune responses of different immune complexes in primary human myeloid cells (which cells to use is an important question and is addressed below). Antibodies to JE complexed with DENV result in mild ADE. Thus, this immune complex sends messages down the tail of the FcR that are very different from a complex formed by a DENV and antibodies from a primary dengue infection. What is the exact effect on iADE, and what is the molecular mechanism that controls response differences, if any?

Role of different myeloid cells
How myeloid cell phenotypes correlate with disease severity and iADE requires more investigation. We must start by going back to human autopsies and identifying macrophage targets for DENV infection using comprehensive cell markers. These investigations are currently in their infancy (16). All human myeloid cells should be studied separately.

Role of different FcγRs in iADE
This is another huge field for research. It should be remembered the DVPS occurs in individuals who circulate myeloid cells that express various FcRs, often on the same cell. Ultimately, iADE needs to be studied using this inherent complexity.

Role of DENV in ADE
Enhanced growth of DENV-2 recovered from severe compared with mild disease in primary human monocytes
The simple experiment as designed in the published paper should be repeated (117).

Differences in ability of DENV-2 strains to be neutralized by DENV-1 antibodies
The actual sites on the virion that mediate these differences in human disease expression are still unknown. Differences in genetic and viral structure between DENV-2 viruses that are and are not associated with epidemic DHF/DSS during a first heterotypic dengue infection are well established. Viral contributions to these differences have not been studied in the appropriate in vitro system, e.g., primary human myeloid cells from flavivirus-naïve individuals.

DENV-2 genetic differences associated with a major increase in disease severity of DENV-2 infection 20 years after infection with DENV-1
What are the kinetics of growth of DENV-2 in the primary human myeloid cell assay system with and without the NS1 amino acid changes?

Genetic differences in DENV-2 isolates recovered from disease of enhanced severity 4 years after DENV-1 and 8 years after DENV-3
The authors have a surfeit of research materials (138). The only sensible approach to studying these phenomena is to test the reagents in the ADE primary human myeloid cell system. This will have to be done on a large scale. Differences in the viral productivity of cells infected using infectious immune complexes should provide outcomes that can be related to any of a number of viral, antibody, or cellular differences.

Efferent
The final scenario that results in DVPS has been amazingly difficult to learn about. Complement split products and key cytokines have been thought to mediate vascular permeability at the same time these factors were thought to produce thrombocytopenia and altered hemostasis. The failure of cortisone administered early to suppress cytokine production or reduce the incidence of vascular permeability was a wake-up call. An alternative mechanism is needed. I suggest that DVPS is a dengue toxin. Dengue NS1 directly causes all of the elements of DVPS—activated complement, thrombocytopenia, altered hemostasis, and vascular damage. As a direct toxin, NS1 should produce its damage in proportion to the concentration of NS1 presented to key systems and tissues. ADE functions directly to increase the concentration of NS1 proportionate to infected cell mass. But why doesn’t DVPS occur earlier in the disease when peak NS1 blood levels are achieved? Why does DVPS suddenly worsen at defervescence? I posit that this is related to the onset of killer T cell activity. T cells damage dengue-infected cells, resulting in a final bolus release of NS1. When this exceeds a threshold, DVPS reaches its critical level of damage. This hypothesis also includes the possibility that NS1-antibody complexes may retain significant toxicity. I challenge the dengue research community to prove me wrong.

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Dengue Antibody-Dependent Enhancement: Knowns and Unknowns


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Dengue Antibody-Dependent Enhancement: Knowns and Unknowns


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