Dengue Research Opportunities in the Americas

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Dengue is a systemic arthropod-borne viral disease of major global public health importance. At least 2.5 billion people who live in areas of the world where dengue occurs are at risk of developing dengue fever (DF) and its severe complications, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Repeated reemergences of dengue in sudden explosive epidemics often cause public alarm and seriously stress health-care systems. The control of dengue is further challenged by the lack of effective therapies, vaccines, and point-of-care diagnostics. Despite years of study, even its pathogenic mechanisms are poorly understood. This article discusses recent advances in dengue research and identifies challenging gaps in research on dengue clinical evaluation, diagnostics, epidemiology, immunology, therapeutics, vaccinology/clinical trials research, vector biology, and vector ecology. Although dengue is a major global tropical pathogen, epidemiologic and disease control considerations in this article emphasize dengue in the Americas.

Dengue fever (DF) and its severe complications, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), are among the most important reemerging infectious diseases globally. At least 2.5 billion people live in the tropical and contiguous temperate areas where dengue occurs, resulting in an estimated 50–100 million annual infections, 500 000 cases of DHF/DSS, and 20 000–25 000 deaths. Sudden, explosive, and repeated epidemics cause public alarm and stress public health control systems (Figures 1 and 2) [1].

The epidemiologic picture of dengue has been worsening, with rebound of its principal mosquito vector, *Aedes aegypti*, in many areas and the rapid global expansion of a secondary vector, *Aedes albopictus*. Dengue prevention by vector control has proven difficult, and there is as yet no vaccine or specific therapy. The pathogenesis of DHF/DSS is unknown; however,

6 decades of increasing cocirculation of multiple dengue virus (DENV) serotypes (DENV-1, -2, -3, and -4) has been associated with progressive global expansion of DHF/DSS. Impending shock can be prevented with fluid and electrolyte therapy; however, early identification of severe cases is difficult. Preventable deaths continue to occur, particularly in areas with few trained healthcare workers. Thus, dengue remains an important potentially fatal epidemic disease that challenges public health, clinical, and research systems at many levels [1].

BACKGROUND IN THE AMERICAS

When and where *A. aegypti* began to transmit dengue viruses is unknown. Phylogenetic analyses of the 4 DENV serotypes and of their principal vector, *A. aegypti*, suggest an origin of the vector in Africa with virus exportation to the Western Hemisphere approximately 400 years ago, in multiple introductions in association with exploration and the slave trade [2]. Recurrent epidemics of dengue-like diseases in the Americas began to be recorded in the early 17th century. The first recognized dengue outbreaks, reported in 1635 on the Caribbean islands of Martinique and Guadeloupe, featured sudden onset of illness and
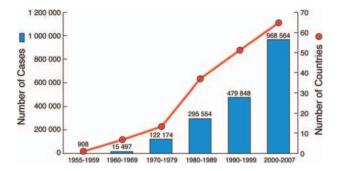
Figure 1. The increasing global dengue problem, 1955–2007. Source: World Health Organization.

Figure 2. Cocirculation of multiple dengue (DEN) serotypes in the Western Hemisphere, 2006–2010. Source: Pan American Health Organization.

symptoms including fever, violent headache, lassitude, and pains in the legs, popularly referred to as *coup de barre* ("beating with a stick" [3]). In 1780, Benjamin Rush described a dengue outbreak in Philadelphia and coined the similar term "break-bone fever" [4]. Dengue was long considered a nuisance disease with low rates of mortality. However, as the 4 serotypes spread globally, reinfections with different serotypes began to be associated with severe forms of the disease. The earliest known cases of probable DHS/DSS were seen in India in the 1870s [5].

CURRENT STATUS OF DENGUE IN THE AMERICAS

Attempts to eradicate *A. aegypti* in the Western Hemisphere achieved considerable success between 1947 and 1970. However, the banning of the insecticide DDT, coupled with the deterioration of public health programs, led to rapid vector reestablishment, associated with population growth, urbanization, and importation of new DENV serotypes and strains. In recent decades this has led to progressive regional increases in dengue. Without a vaccine or specific treatment, disease control has relied on general public health measures, including surveillance, education, early recognition of cases, and vector control; however, many countries at risk for dengue lack adequate public health resources.

The wide distribution and simultaneous circulation of the 4 dengue serotypes in several countries in the Americas is a warning sign for further spread of severe forms of the disease associated with secondary infections (Figure 2). Unlike the situation in most Asian countries, where dengue is primarily a pediatric infection, in the Americas dengue predominantly affects adults, although there have been increasing reports of dengue fever and DHF/DSS in children.

In 2003, the Ministers of Health of the Pan American Health Organization approved a resolution to implement an Integrated Management Strategy for the Prevention and Control of Dengue, combin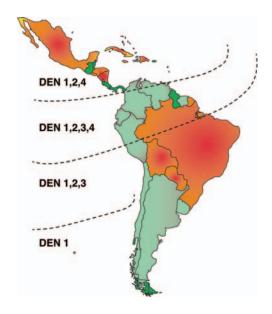ing such key elements of disease response as epidemiologic surveillance, vector control, environmental control, patient care, laboratory testing, and communication [6]. National reference laboratories have been added to the Dengue Laboratory Network to work closely with regional World Health Organization (WHO) collaborating centers in Argentina, Brazil, the English-speaking Caribbean, Cuba, and Puerto Rico. The network assesses laboratory capabilities, conducts proficiency testing, and helps prepare research agendas and capacity-building plans. Such relationships are important but do not replace the urgent need for research aimed at providing better tools for prevention and treatment.

VIRAL REPLICATION

First isolated by Hotta in 1943 and Sabin in 1944, DENVs are enveloped, positive-strand RNA viruses belonging to the family Flaviviridae. Mature viral particles are composed of genomic RNA complexed with several copies of capsid protein, surrounded by a lipid bilayer containing 180 copies each of the membrane (M) and envelope (E) proteins. DENV-infected cells release whole virions as well as significant amounts of noninfectious immature virus particles containing uncleaved precursor M protein (prM) [7].

The primary cellular targets of DENV infection are still unknown. However, studies conducted in human autopsy tissues and in a mouse model of dengue infections have identified monocytes, macrophages, and mature and immature dendritic cells as some of the main cells targeted by dengue infection [8]. The precise mechanism of dengue cell entry is unknown. Several different putative receptors in humans and

in mosquitoes have been implicated in mediating viral attachment and entry in different cell types, including DC-SIGN, CD209, mannose receptor, and C-type lectin [9]. DENV infects cells by clathrin-mediated endocytosis [10]. Acidification of the endosome induces specific structural changes in E glycoproteins that trigger fusion of viral and endosomal membranes and release of viral nucleocapsids into the cytoplasm. This process utilizes as cofactors cellular anionic lipids, synthesized only in late endosomes, to ensure genome release into the correct cellular compartment [11].

Recent advances in understanding mechanisms of genome translation and replication suggest opportunities for antiviral drug design. An example is the discovery of unique interacting RNA elements at the 5′ and 3′ genome ends that lead to genome circularization, which is required for genome replication [12]. DENV replication also induces rearrangement of cellular membranes of the endoplasmic reticulum (ER) to form specialized intracellular structures for RNA replication and virus assembly [13]. Infection induces relocalization of the cellular enzyme fatty acid synthase (FASN) to these replication sites and stimulates FASN activity and lipid biosynthesis. This results in the accumulation of ER-derived lipid droplets, which seem to be required for genome encapsidation [14]. An FASN inhibitor that decreases ER-derived lipid droplets inhibits production of viral particles by 100–1000-fold [14], suggesting that licensed inhibitors of lipid synthesis might have a role in dengue therapy.

IMMUNE RESPONSES

Filling gaps in knowledge about viral replication will likely also elucidate the viral particle types and viral products encountered by the immune system. DENV encodes several proteins that antagonize components of the innate immune response. These include NS5, which targets for degradation STAT2, an important component of the type I interferon pathway [15]. Binding and degradation of STAT2 is species-specific, which may explain why DENV replicates poorly in immunocompetent mice but grows well and causes disease in mice without interferon response genes [16, 17]. Lack of an immunocompetent animal model for studying dengue has delayed the understanding of disease pathogenesis and the evaluation of therapies and vaccines. Research leading to a better understanding of restriction factors for dengue replication in mice could lead to a transgenic, immunocompetent mouse model that mimics dengue disease pathology.

Many observations suggest that immune responses to dengue are determinants of disease severity. Epidemiological studies have shown that most DHF/DSS cases occur either during second dengue infections with different dengue serotypes, or in infants born with maternal anti-dengue immunoglobulin G that has declined to subneutralizing levels [18]. There are 2 main theories to explain the mechanism of

dengue immunopathogenesis. The first is that antibodies raised against the primary infection do not neutralize the virus causing the second heterologous infection, but instead promote viral replication by antibody-dependent enhancement (ADE). In ADE, nonneutralizing antibody complexed to virus increases viral uptake by Fc receptor–bearing cells [19]. ADE has been recapitulated for dengue both in tissue culture and in mouse and monkey models [20, 21].

Another theory of dengue immunopathogenesis proposes that memory T cells reactivated by second heterologous infections express altered cytokine levels contributing to plasma leakage and increased disease severity [22]. This theory is consistent with clinical studies and animal research, although it does not easily explain DHF/DSS in infants. Both phenomena, as well as other factors including virus virulence, and genetic/nutritional host factors, may play a role to differing degrees in the development of severe dengue.

DENV-specific antiviral antibody responses are directed mainly against E, prM, and NS1 [23]. Nonneutralizing antibodies raised against prM are highly cross-reactive against all DENV serotypes, and potently promote ADE in tissue culture. The prM antibodies are increased in symptomatic secondary dengue infections [24] and constitute a large fraction of the total antibody repertoire in DHF [25]. Normally, noninfectious immature virus particles can become highly infectious in the presence of anti-prM antibodies [9], consistent with the possibility that during secondary infection preexisting, cross-reacting anti-prM antibodies that bind both virions and immature virus particles might increase disease severity by increasing the viral load. The therapeutic option of monoclonal antibodies might logically be discarded due to fear of eliciting ADE. However, mutations in antibody Fc regions totally eliminate ADE in mice, indicating that it may be possible to design safe and effective treatment with neutralizing monoclonal antibodies. Mutations in antibody Fc regions eliminate ADE in mice [21, 23], indicating that single specificities or "cocktails" of neutralizing monoclonal antibodies might be therapeutically useful. A better understanding of the immunopathogenic mechanisms underlying dengue is crucial for developing safe vaccines and therapeutics that will not potentiate severe disease following natural infection.

DENGUE DIAGNOSTICS

Sensitive and specific assays to identify DENV infection are important for both patient care and epidemiologic studies. Tests that measure viral RNA or antigens are generally useful at the time of symptom onset, while tests that measure immunoglobulin M (IgM) become useful within 4–6 days thereafter. A combination of these types of assays would improve both sensitivity and specificity (distinguishing dengue from conditions with similar clinical signs and symptoms, such as

influenza) (Figure 3). Although several kits are in development for clinical diagnosis by virus culture, or by nucleic acid, NS1, or IgM detection, no test is yet licensed for point-of-care diagnostics. The Centers for Disease Control and Prevention and WHO have collaborated in the development of specimen panels, as well as guidelines for the design, conduct, and validation of diagnostic assays for sensitivity, specificity, and reliability [26]. Although few instances of transfusion-transmitted dengue have been reported, the American Association of Blood Banks has listed dengue as 1 of 3 priority emerging infections for future blood screening [27].

Because DHF and DSS can usually be successfully treated with early administration of fluids and electrolytes, and because tertiary care facilities are often limited in resource-poor settings, it is important to distinguish those few patients with DF who will progress to DHF/DSS from the majority who will recover uneventfully. This has fueled an intense search for early-onset biomarkers that reliably identify imminent severe disease [28]. Research has centered on factors that seem to be involved in pathologic mechanisms, such as elements of the complement system, the innate immune system, cytokines, and easily measured blood components. Both protein and nucleic acid detection technologies are being utilized on routine patient specimens such as blood cells and serum.

DENGUE TREATMENT

Although deaths from DHF and DSS can almost always be prevented by early institution of fluid management and supportive care, there remains a need for specific therapies, especially for patients who present in advanced stages of disease. A major challenge to drug development is the lack of an immunocompetent animal model that adequately replicates human dengue pathology. In humans, evidence that DHS and DSS are associated with higher viral titers and longer durations of viremia [29] does, however, support the clinical utility of antiviral drugs. Of note, drugs for chronic infection with the related flavivirus, hepatitis C are being energetically pursued as possible therapies for dengue. Other promising therapeutic approaches include (1) direct-acting antiviral drugs that target virally encoded functions, (2) drugs that target host functions essential for viral replication, and (3) drugs that target dengue-associated pathology, such as vascular leakage. Among virally encoded targets are enzymes such as RNA polymerase, helicase, protease, and methyltransferase. Viral structural proteins, such as the E glycoprotein, can serve as a target for drugs that block cell receptor binding and fusion (Figure 4). For example, an inhibitor of viral entry reduced both dengue and yellow fever virus replication, and was also effective in an in vitro model of ADE [30]. Few direct-acting antiviral drugs have progressed beyond early preclinical studies; however, despite the potential for selection of resistance mutations, they offer the promise of potency without toxicity.
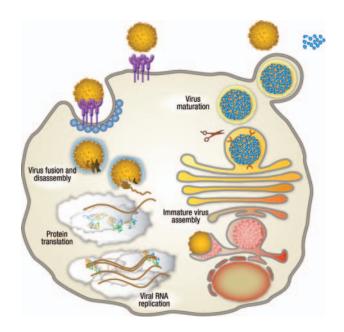

Figure 3. Dengue virus life cycle and potential antiviral targets. Courtesy of Richard Kuhn, Purdue University. Adapted from the original image.

Like all viruses, DENV relies on its host to provide many replicative (and potentially pathologic) functions. Therapies directed at host processes have the theoretical advantages of (1) being unlikely to lead to drug resistance and (2) potentially having broad-spectrum activity against different viruses utilizing the same host pathways. The redundancy built into many host functions provides hope that the host can circumvent drug toxicity by substituting 1 or more alternate pathways for a specific function required for viral replication. One study using RNA interference screening to compare human and mosquito host factors influencing DENV replication [31] identified 42 insect and human homologues involved in
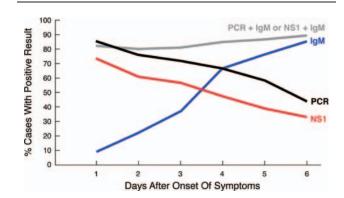

Figure 4. Sensitivity of dengue diagnostic tests. Based on Centers for Disease Control and Prevention (CDC) surveillance data and validations of commercial kits. Courtesy of Jorge Munoz-Jordan, CDC. IgM, immunoglobulin M; PCR, polymerase chain reaction.

dengue replication. Host functions important for DENV replication include enzymes involved in nucleic acid synthesis, lipid metabolism, kinases, and protein chaperones. For some of these—such as the kinases—specific inhibitors already licensed by the Food and Drug Administration for other indications could prove useful for dengue treatment.

A third strategy, therapies that target specific host pathologic processes resulting from infection, has also been explored. DHF and DSS occur in association with breakdown of host control over vascular stability. Virus-associated inflammation can result in the release of multiple cytokines that disrupt vascular homeostasis. Although lack of an appropriate animal model for vascular leakage is a serious drawback [32], a drug that interrupts capillary dilation and the formation of capillary "pores" could be beneficial. Moreover, as vascular instability is the end result of infections caused by many different microbes, such a strategy might be broadly applicable.

VECTOR RESEARCH

The 2 primary mosquito species that transmit dengue among humans are A. aegypti and A. albopictus. Of these, the former is considered most important because it is an efficient DENV vector prevalent in tropical areas of the world where dengue is endemic. Every aspect of this important vector, from its ecology and behavior to its metabolic pathways and innate immune responses, has been studied extensively.

Research on virus/vector interactions has progressed at a rapid pace since sequencing of the A. aegypti genome in 2007 [33]. Advances include the identification of genes that can be used to develop transgenic insect strains to potentially prevent transmission of dengue to humans. For example, genes involved in Aedes blood digestion and egg development can be targeted to disrupt mosquito feeding and reproduction. Another potential gene target influences female mosquito flight. Transgenic mosquitoes and mosquitoes with genetically modified symbionts have recently been field tested and shown to have a deleterious effect on both mosquito survival and survival of the virus within the mosquito [34].

Population biology research on A. aegypti in different geographic regions has enhanced the knowledge of vector competence of different mosquito strains for different DENV serotypes. Such studies also increase the understanding of how insecticide resistance emerges and spreads. Genomic analysis of detoxification genes and the mapping of genetic regions controlling insecticide resistance in A. aegypti could potentially enhance control efforts [35]. Improved understanding of mosquito olfaction and host-seeking is key to developing effective traps and repellents. Studies on mosquito ecology, feeding, mating, and oviposition support the development of more effective control strategies. For example, the implementation of casa segura ("safe house"), a program to promote use of insecticide-impregnated curtains to reduce mosquito numbers in houses, as well as the use of lethal oviposition traps, should help control dengue in resource-constrained endemic areas [36].

Studying the epidemiology of dengue in endemic sites is essential to understanding the seasonality of transmission and the risk of infection. Epidemiologic studies must include multidisciplinary approaches that consider the degree of human disease expression (subclinical cases may be important), vector abundance, vector distribution and seasonality, and human behavior. Mathematical models that can inform interventions for control are being developed based on field-collected mosquitoes and epidemiological data that include human factors.

DENGUE VACCINES

The need for effective vaccines to control dengue is urgent and compelling in light of the increasing emergence of dengue and expansion of areas of endemicity. Efforts toward vaccine development have been ongoing for more than 7 decades. Many factors underlie the difficulty of developing dengue vaccines, including (1) the lack of both an immunocompetent animal model for human disease and a defined immune correlate of protection, (2) the need to simultaneously induce immunity to 4 distinct serotypes, and (3) the obligation to proceed with caution because of concern that "incomplete" immunity may be a risk factor for subsequent severe dengue disease. Despite these challenges, 2 types of tetravalent dengue vaccines, live attenuated and nonreplicating, are currently in clinical development (Table 1) [37].

Live attenuated vaccines have advanced the furthest in clinical studies. Phase 2 and 3 studies of a chimeric tetravalent vaccine with a yellow fever 17D backbone (Sanofi Pasteur, CYD) are under way at numerous sites. Data from these studies have confirmed the safety and immunogenicity after 2 doses in flavivirus-immune subjects, and after 3 doses in flavivirus-naive subjects [38]. Monovalent preparations of another live attenuated vaccine developed by intramural researchers from the National Institute of Allergy and Infectious Diseases have been evaluated, and tetravalent formulations are currently under study. Preliminary results from clinical trials of the tetravalent formulations indicate that these vaccines are safe and can induce trivalent or tetravalent neutralizing antibody responses in 75%–90% of trial volunteers after a single dose [39]. Phase 1 studies of an additional tetravalent chimeric vaccine (DEN-2 PDK-53 background; Inviragen) are under way in adults in the United States and Colombia to evaluate both low- and high-dose formulations [40]. A nonreplicating DEN-1 recombinant E protein subunit vaccine (Merck/Hawaii Biotech) has also been evaluated in a phase 1 trial, demonstrating safety and immunogenicity when formulated with aluminum hydroxide [41]. Reformulation as a tetravalent product is under way.

Table 1. Dengue Vaccines in Clinical Development

Vaccine Type	Developer(s)	Development Stage (as of November 2011)	Recent Reference
Live attenuated	Sanofi Pasteur/Acambis	Phase 3	[38, 49]
Live attenuated	GSK/WRAIR	Phase 2 (suspended)	[50]
Live attenuated	NIAID, NIH	Phase 1	[39, 51]
Live attenuated	Inviragen/CDC	Phase 1	[40]
Subunit	Merck/Hawaii Biotech	Phase 1	[41]
DNA	NMRC	Phase 1	[43]

Abbreviations: CDC, Centers for Disease Control and Prevention; GSK, GlaxoSmithKline; NIAID, National Institute of Allergy and Infectious Diseases; NIH, National Institutes of Health; NMRC, Naval Medical Research Center; WRAIR, Walter Reed Army Institute of Research.

Numerous nonreplicating dengue vaccine candidates are currently in preclinical development [42], including DNA vaccines [43], inactivated virus [44, 45], and virus-vectored vaccines. Alphavirus replicon vectors such as Venezuelan equine encephalitis virus have been engineered to infect cells and express DENV structural proteins in a propagation-defective replication cycle. Such particles are immunogenic and protect against DENV challenge in monkeys; evaluation of a tetravalent formulation is under way [46]. Alternatively, E and prM proteins from all 4 DENV serotypes can be expressed from independent promoters in 2 replication-deficient adenovirus vectors. In addition, the codons of the DENV antigens have been optimized for increased expression in human as opposed to mosquito cells. Vaccination of rhesus macaques with these particles induces a balanced antibody response and protects against all 4 DENV serotypes [47]. Another approach to optimizing antigen immunogenicity analyzed the E genes of multiple strains of each DENV type. A consensus sequence was used to create a computationally optimized, broadly reactive E protein antigen (COBRA), similar to the broadly cross-reactive antigens prepared for influenza virus [48]. Such COBRA E antigens expressed in their natural conformations on subviral particles elicit strong antibody responses in mice against all 4 serotypes (unpublished data).

FUTURE DIRECTIONS

There are many promising areas for future research and approaches that seem to offer the greatest promise of return on investment. Interdisciplinary studies of the interface between DENV and its human and mosquito hosts are urgently needed, as this research information is essential to the design and development of safe and effective vaccines and treatments. It is also important both for the development and evaluation of accurate diagnostic assays for both individual patient care and epidemiologic endeavors to control vector populations and disease outbreaks.

The importance of integration of clinical with basic research, enhanced collaborations across seemingly unrelated disciplines, and the inclusion of studies involving genomics and bioinformatic tools is an important area of emphasis. Building on the several ongoing global collections of viruses, viral sequences, and clinical specimens (www.viprbrc.org, accessed 15 June 2012), efforts should be strengthened to correlate viral genome sequences with geographic sources, host antibody status, disease severity, and host genetic elements that influence replication and pathogenesis. International collaborations on the identification and preparation of field sites for studies of new diagnostics, vaccines, and therapeutics are also needed. Similarly, cross-disciplinary collaborations for the study of vector control measures, both behavioral and chemical, may be as essential to disease control as the development of vaccines and drugs. Thus, the ultimate control of dengue will require collaborative research programs undertaken by investigators representing many disciplines and an integrated multidisciplinary and multinational research program.

Notes

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