The Current Situation of Dengue Research

TAKESHI KUROSU*

*Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

Dengue is a mosquito-borne disease that is caused by the dengue virus (DENV) and that represents an important public health problem in tropical and subtropical countries. Because it is carried by mosquitos, there were no cases of domestic infection in Japan for the last 70 years. However, 162 persons were infected with DENV in Japan last year, possibly due to the changes in climate or human migration. Research on dengue has a long history, but many important questions have not yet been answered. For instance, it is still unknown why severe dengue occurs when a patient is secondarily infected with a different serotype of DENV. It is believed that severe dengue is the result of vascular leakage induced by an excessive host defense reaction, but it remains unknown what triggers the change in vascular permeability. DENV-infected patients often show thrombocytopenia, a typical symptom of dengue, but it is not known what causes the thrombocytopenia and if there is a correlation between thrombocytopenia and vascular leakage. This review describes the features of DENV infection and the current state of developing dengue vaccines and treatments.

Key words: dengue fever, dengue hemorrhagic fever, tropical diseases, mosquito-borne infection

Introduction

The first recorded case of probable dengue fever was described in a Chinese medical encyclopedia from the Jin Dynasty. The text, written in AD 265-420, associated the disease with a flying insect (Dengue Virus Net, <http://www.denguevirusnet.com/history-of-dengue.html>). The first recognized dengue epidemics occurred in Asia, Africa, and North America in the 1780s. The first confirmed case was reported in 1789 by Benjamin Rush, and he named as "breakbone fever" because of the symptoms of myalgia and arthralgia. The dengue virus (DENV) was first isolated by Japanese researchers in 1943¹⁾. Since then, many DENVs have been isolated using mosquitoes, suckling mice, and cultured cells. Recently, a mosquito cell line, which was also established by a Japanese researcher²⁾, has become a commonly used tool for virus isolation because of its high virus isolation rate and easier handling.

Clinical features

Dengue is clinically categorized into two types that differ in severity. One type is dengue fever (DF), which causes fever, rash, myalgia, joint pain, abdominal pain, and thrombocytopenia. Another is dengue hemorrhagic fever (DHF), a more severe form of the disease that causes hemorrhagic symptom, hepatic injury, and severe thrombocytopenia. Although severe dengue is called dengue "hemorrhagic" fever, hemorrhage is not the basis for the severity but the result of increased vascular permeability. In patients with DHF, an accumulation of fluid in the abdominal and pleural cavities is often observed, and hemorrhage can even occur in brain in severe cases. DHF has a fatality rate of about 1% when patients are not properly treated.

In the case of the most severe form of DHF, patients die of hypotonic shock because of the loss of body fluids from the blood circulation; therefore, the maintenance of body fluid balance improves

Takeshi Kurosu

*Present Address: Department Virology 1, National Institute of Infectious Diseases
4-7-1 Gakuen, Musashimurayama-shi, Tokyo 208-0011, Japan

TEL: +81-42-561-0771 E-mail: kurosu@niid.go.jp

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survival. Once patients survive the shock syndrome, they do not have any sequelae. Compared to other viral hemorrhagic fevers, such as Ebola virus infection, DHF is a relatively curable disease. During a dengue epidemic in Japan, there was only one DHF case. Indeed, most dengue-infected patients experienced only a flu-like illness that completely resolved within 7–10 days. However, dengue is a leading cause of hospitalization and death among children in South East Asia³⁾ because the absolute number of patients with dengue is huge in many of these countries.

A unique feature of DHF is that vascular leakage occurs during the recovery period. Vascular leakage does not occur at the peak of viremia or fever, but only when patients start recovering from dengue at the time of defervescence⁴⁾. Vascular leakage starts suddenly and ends rapidly in cases with a positive outcome, taking just 24–36 hours from start to recovery. Patients usually visit the clinic 1 or 2 days after the onset of fever. During this period, patients have the highest viremia while their platelet counts are already dropped. However, until they experience increased vascular permeability, there is no clear sign that distinguishes between DF and DHF, suggesting that vascular changes are not predictable. This is the most challenging problem for clinicians, who need to decide whether to treat patients or to monitor for vascular leakage by measuring hematocrit values when they suspect severe dengue. Because there is no marker to predict vascular leakage, clinicians need to judge from clinical symptoms and blood test results. They mainly pay attention to platelet counts, and a sudden drop in platelet count or a diagnosis of severe thrombocytopenia at the first medical examination is regarded as a sign of severe disease. There is a negative correlation between platelet counts and severity, but a low platelet count does not always lead to severe disease (vascular leakage)⁵⁾. Moreover, most patients recover without experiencing critical vascular leakage or hemorrhage. Therefore, there is a risk that hospitals treat patients unnecessarily, which is not economically or administratively effective because these patients occupy the limited number of beds available. To avoid this situation, a reliable marker molecule that can predict vascular leakage must be found.

Figure-1 Primary infection vs. secondary infection

Another unique feature of dengue is that severe disease often occurs in patients secondarily infected with other serotypes of dengue different from that of the primary infection. Dengue has four serotypes, DENV-1–4, and all four serotypes can cause severe dengue. For example, once a person becomes infected with one type of DENV (e.g., DENV-1), he or she will never be infected again with same serotype of DENV (Figure-1). However, this person could still become infected with other serotypes of DENV (e.g., DENV-2, -3, or -4). This is called secondary infection. In these cases, symptoms are often more severe than in the primary infection. A hypothesis to explain this greater severity is that the secondary infection increases viral production through antibody-dependent enhancement (ADE). Indeed, higher viremia has often been observed in patients with secondary infection or severe dengue. In a secondary infection, antibodies (Abs) from the primary infection do not have neutralizing effects. Because target cells for dengue, such as monocyte/macrophages, express Fcγ receptors (FcγRs) on their surface, immune complexes containing DENV and non-neutralizing anti-DENV Abs (virus–Ab immune complexes) could efficiently enter these cells through FcγR (Figure-2). As a result, patients have a higher viral production. However, this is not the end of the story. Higher viral production induces a stronger immune response, such as a cytokine storm, excessive complement activation, or massive production of non-neutralizing Abs, which may increase vascular permeability.

There is a unique feature observed in DENV-infected patients that is thought to be related to vascular leakage and severe disease. At the beginning of a DENV infection, the soluble form of

Figure-2 Secondary infection with DENV

the DENV nonstructural protein 1 (NS1) is solely found in the circulating blood (Figure-2). This is unusual because nonstructural proteins usually function in viral replication in the cytoplasm. Nonstructural, in fact, means not included in the viral particle, and so not usually released from cells. At present, we do not know why NS1 is released into the blood circulation. Nonetheless, this soluble form of NS1 (sNS1) in patients' sera is now used as a diagnostic marker for DENV infection because sNS1 is present in blood at an early stage of disease progression⁶⁾. In fact, most of the currently available kits for an early diagnosis of dengue assay sNS1 levels.

The causative mechanisms of the two major symptoms, vascular leakage and thrombocytopenia, remain unknown. It is very difficult to know their pathogenesis because it is likely that the symptoms of severe dengue are the result of an excessive host response, as described above. For example, pleural and abdominal effusions are often observed in patients with DHF, but still we do not know in which organs the fluid leakage was triggered. Analyzing the virus will not provide a final answer about the pathogenesis. The best way to understand the pathology of DHF is to study patients; however, there is an ethical limitation to this kind of

research. In the next section, the possible mechanistic hypotheses for severe dengue are discussed.

Vascular leakage

Various types of inflammatory reactions can increase vascular permeability. Inflammation is a protective immunovascular response that aims to eliminate the initial cause of tissue damage triggered by various factors, including infection. However, a state of inflammation that lasts too long or affects the whole body can injure the body. In the case of dengue, virus infection increases vascular permeability as described above. Presumably, the changes in vascular permeability result from the virus-induced increases in levels of cytokines, chemokines, soluble chemical mediators, or activated components of the complement system. Although studies have identified several candidate molecules that may be responsible for the vascular leakage in DENV-infected patients, we still do not know which is the most likely agent.

1. Cytokine hypothesis

Several cytokines and chemokines, such as TNF- α , IL-6, IL-8, IL-10, IL-12, IFN- γ , and MCP-1, have been reported to be elevated in severe cases of

dengue⁷⁾⁻⁹⁾. Elevation of TNF- α , which is well known for its ability to induce vascular leakage, is the most commonly observed change. This is true even in a mouse model of lethal dengue, where types I and II interferon receptor knockout mice had a sudden elevation of TNF- α in the morbid stage of disease, as observed by several groups including our own¹⁰⁾. Treatment with an anti-TNF- α neutralizing antibody prevented vascular leakage and protected mice from lethal infection. These results suggest that massive production of TNF- α may be a critical factor in severe dengue.

2. Complement hypothesis

High levels of circulating immune complexes (CICs) can also be detected in DENV-infected patients¹¹⁾. Complement activation is thought to be extremely high because there is an apparent consumption of complement. At least two types of immune complexes exist in DENV-infected patients: one is composed of the DENV particle and anti-DENV envelope (E) protein Abs, and another is composed of the DENV NS1 protein and anti-NS1 Abs. Since high levels of sNS1 seem to be related to severity, several groups have studied the role of immune complexes containing sNS1 in plasma leakage. Indeed, immune complexes activate the classical pathway of complement, which may cause vascular leakage.

The effects of immune complexes on the body depend on their affinity to organs and on their size. Small CICs usually last longer in the blood circulation and sometimes cause vasculitic syndrome by a type III allergic reaction. The size of CICs containing NS1 is thought to be small compared to the viral particle because NS1 forms no more than a hexamer, while the viral particle is large because it contains several hundreds of C, M, and E protein molecules. The activation of complement by DENV infection can potentially increase vascular permeability. Although recent research has focused on cytokines and soluble cellular mediators, there may be still potential involvement of CICs or complement in vascular leakage.

3. Chemical mediator hypothesis

In addition to the factors described above, chemical mediators may also have a role in vascular leakage. Histamine has been studied in the context

of dengue since the 1970s because it is a strong inducer of vascular permeability. Indeed, dengue patients have raised levels of blood and urinary histamine, produced by mast cells and basophils. However, thus far there is no direct evidence to prove a link between histamine and vascular leakage. Other chemical mediators may also cause vascular leakage, but they have not yet been studied in detail.

Thrombocytopenia

Severe thrombocytopenia is not sufficient to cause hemorrhage or vascular leakage; however, severe thrombocytopenia is observed in the most severe cases and there is a negative correlation between platelet counts and severity. For this reason, clinicians monitor platelet counts in dengue patients as an empirical way to predict shock syndrome. Here, I will discuss the hypotheses that may explain severe thrombocytopenia in DENV-infected patients.

1. Bone marrow suppression

Severe thrombocytopenia has been observed in DHF patients since the first studies of dengue⁵⁾. Thrombocytopenia is thought to be due to bone marrow (BM) suppression. Indeed, BM-derived cells are susceptible to DENV infection, although the target cell has not been clearly identified. Although the two symptoms, vascular leakage and thrombocytopenia, are typically observed in severe dengue, the critical difference between them is the timing of their onsets. Vascular leakage occurs during recovery period, when viremia starts to decrease and body temperature returns to normal, as described above. On the other hand, thrombocytopenia starts together with high fever in the acute phase. At the beginning of DENV infection, patients already have high levels of thrombopoietin, which stimulates megakaryocytes to produce platelets after sensing a drop in platelet numbers¹²⁾. This observation suggests that an event triggering thrombocytopenia starts even earlier than the manifestation of thrombocytopenia in the peripheral blood because the patient's body is already trying to recover from the acute phase. Therefore, there may be no direct connection between thrombocytopenia and vascular leakage because the

timings are different.

Regarding the target cells of DENV in BM, Clark *et al.* recently demonstrated that CD61-positive cells derived from human BM are highly susceptible to DENV¹³⁾. The integrin CD61 is a surface marker specifically expressed by platelets and their megakaryocyte precursors. Although further research is needed, this finding seems to fit with previous observations. In addition, we have observed that DENV preferentially infects CD11b- and CD11c-positive cells in mouse BM. Furthermore, an engineered chimeric virus, which carries the prM and E (prME) genes of DENV within the Japanese encephalitis virus, caused complete loss of megakaryocytes and elimination of thrombocytopenia in mice (unpublished data). Therefore, DENV infection is likely to directly or indirectly disrupt megakaryocytes.

2. Phagocytosis

Although BM suppression is an attractive story to explain thrombocytopenia in dengue, there is an additional hypothesis about this symptom. Part of the thrombocytopenia may be due to the phagocytosis of platelets by macrophages¹²⁾. DENV particles are thought to attach to platelets directly or indirectly through anti-DENV Abs, because human platelets express Fc γ receptors. As a result, platelets in dengue patients may be phagocytosed, explaining, at least in part, the observed thrombocytopenia. Taken together, although this work is preliminary, BM seems to play an important role in the thrombocytopenia observed in dengue.

Vaccines and antiviral drugs

1. Dengue vaccines

Many groups have been developing vaccines against DENV¹⁴⁾. Currently, the most advanced vaccine is a chimeric, live-attenuated vaccine named ChimeriVax Dengue Tetravalent Vaccine, derived from the yellow fever virus (YFV) vaccine strain 17D. YFV-17D has been used in the world and its efficacy has been proved. ChimeriVax Vaccine viruses carries the prME genes of DENV, while the rest of the genome, including genes encoding the capsid, NS1-5, and the 5'- and 3'-UTRs, is derived from YFV-17D. The protective effect of vaccination is probably due to humoral

immunity against E protein expressed on the surface of virions.

A phase IIB clinical trial performed by Sanofi Pasteur partly demonstrated the efficacy of ChimeriVax vaccine; however, it also showed little protection against DENV-2, although sera from vaccinees had neutralizing Abs to this serotype¹⁵⁾. This observation provided some very important information. There is an on-going debate on why vaccination with ChimeriVax showed no protection to DENV-2, and four hypotheses have been put forward: 1) DENV-2 vaccine strain did not match with the DENV-2 strain currently prevalent in Thailand, where the study was conducted. 2) The four viruses interfered with each other, and DENV-2 failed to replicate in the vaccinees' bodies as a result. 3) DENV-2 escaped from the vaccine because it was the major serotype in that epidemic and major virus strains have a greater chance of accumulating mutations that help them escape from immunological pressure. 4) In addition to humoral immunity, cellular immunity plays an important role in protection. In fact, although prM and E proteins of DENV induced cellular immunity, most T-cell epitopes are located in the NSs, which, in the case of ChimeriVax vaccine viruses, are derived from the YFV vaccine strain. If cellular immunity is key to clearance of DENV, the vaccinees' immune systems might fail to eliminate DENV because the NS region of ChimeriVax Dengue vaccine virus does not match that of DENV. However, it is unclear how important cellular immunity is for the protection against DENV.

There was a big concern that vaccination might cause ADE. If the vaccine failed to induce sufficient immunity, non-neutralizing Abs or very low Ab titer could result. So far, ADE has not been observed after vaccination with ChimeriVax Dengue vaccine. However, this issue must be followed-up in the future because the production of Abs decreases in the long term. In vitro and in mice, ADE occurs when a low level of anti-DENV Abs is introduced (unpublished data). We need to know if the level of Abs can be maintained for more than 10 years.

2. Antivirals against DENV

Unfortunately, development of an antiviral drug against DENV is far behind the progress being made with vaccines. There is no effective, approved

drug against DENV, although several research groups and companies have been struggling to find a novel treatment for DENV over the past few decades. As for other viruses, several steps of the DENV replication life cycle can be targeted, such as viral entry, viral RNA synthesis by RNA polymerase, and maturation of the viral polyprotein by viral protease. Because of the success achieved with an anti-HIV-1 protease drug, dengue researchers are endeavoring to find antivirals against DENV protease. My colleagues and I have identified a potential antiviral drug called SK-12 that targets the viral protease;¹⁶⁾ however, it is still under development.

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