

Literature review:

Epidemiology

Dengue disease is endemic in most tropical and subtropical regions around the world, occurring in South-East Asia and Central America. Dengue hemorrhagic fever (DHF) was first recognized during the dengue epidemics in the Philippines and Thailand and has now become one of the leading causes of hospitalization and death among children. In 2006, the World Health Organization, (WHO) estimated that more than 2.5 billion people are at risk of dengue infection. This leads to approximately 50-100 million new cases of DF and 250,000 to 500,000 cases of DHF are reported each year (WHO, 2006). Demographic changes such as population growth, increased travel and rapid urbanization have resulted in inadequate wastewater management systems and lack of effective mosquito control. These changes, which lead to the spread of dengue virus in populated areas, have resulted in one of the most uncontrolled mosquito-borne viral diseases in the world. Apart from demographic factors, limited financial and human resources have also resulted in poor management and treatment of dengue infections.

The first epidemic of DHF reported in Southeast Asia occurred in 1954 in Manila, Philippines (Malavige *et al.*, 2004). Since that time, epidemics have been reported in nearly all regions of Southeast Asia. Furthermore, the incidence of DHF has increased five fold since 1980 (Malavige *et al.*, 2004). In the Eastern and Southern regions of Asia, epidemics of dengue infection have been less severe when compared to epidemics in the Southeast Asia. At present, Japan has not reported any cases of DF or DHF since World War II. The first epidemic to occur in the Americas took place in Cuba during 1977-78. Since then, it has been projected that the current DHF epidemiological trend of occurrence in the Americas, is expected every three to four years, with increasing cases per epidemic (Malavige *et al.*, 2004). Implementation of dengue control and research, has reduced the occurrence of outbreaks, however, significant outbreaks of dengue infection still persist every five to six years. When looking at dengue epidemics globally, prevalence of dengue infection has also grown dramatically in recent years, with the spread of disease to more than 100 countries worldwide, with emphasis in Southeast Asia (Malavige *et al.*, 2004).

Dengue fever was typically acknowledged as a childhood disease, as it is the main cause of pediatric hospitalization in Southeast Asia. However, there is evidence showing the increasing trends of DHF disease among older age groups in last 30 years. Studies in Asia using surveillance data reports indicated an increasing age of infected patients with DHF (Guha-Sapir and Schimmer, 2005). Hospital based studies have also reported similar results of increasing trends of DHF infection rates among adult populations. In addition to the shift in modal age of dengue infection, race-related susceptibility to dengue has also been observed in a few studies conducted (Guha-Sapir and Schimmer, 2005). Severe dengue disease was observed less frequently in individuals of African origins, which may suggest the inherent resistance gene in black populations compared to other racial populations. Historically, dengue infection has been reported as occurring predominantly among urban populations where the density of dwellings and short flying distance of vector create the desired condition for transmission (Guha-Sapir and

Schimmer, 2005). However, the spread of dengue infection had been reported in rural areas as well due to increased transport contact, mobility, and spread of periurbanization.

Population at risk

It is believed that proper immunization against Dengue virus infections can be effectively done via a live attenuated vaccine. The risks to using a live attenuated virus as a vaccine are generally higher than other means of vaccine types due to their ability to revert to more virulent virus strains (Blaney et al., 2004). The populations at risk include individuals living in areas that experience epidemics of dengue infection especially in tropical and sub-tropical regions, (mainly South and Southeast Asia, Central and South America, and the Caribbean). Since case fatality and hospitalization rates due to dengue infections are highest among infants and the elderly, these individual are more susceptible to dengue infections. DHF is also reported to be more sever among females although there has been no evidence that dengue virus is a gender specific disease. Individual who are immune compromised due to other chronic illnesses are more susceptible to DHF as the serotype DEN-2 is capable of replicating better within peripheral blood mononuclear cells from asthmatics than non-asthmatics. Possible racial differences in susceptibility to dengue infection may put individuals of Asian origin at higher risk than individual of African ethnic group (Malavige *et al.*, 2004).

The vaccine also poses an economical risk, as the demographics effected are heavily plagued with dengue epidemics, and do not have adequate financial means to treat DF/DHF. The problem in administering a live attenuated virus such as this would be the cost of vaccine storage, refrigeration, transportation of the vaccine to areas of need, and personnel to administer the vaccine intravenously to at risk populations (Blaney *et al.*, 2004).

Current Treatments:

There is no effective vaccine available against dengue viral infections. Prevention and control of dengue infections depend largely on preventing the man-vector combat (Malavige *et al.*, 2004). Implementation of environmental control, biological control, chemical control, and active case surveillance are also effective measures that have been taken to control and manage dengue infection.

Prevention methods used to lower the transmission of Dengue fever have been successful by controlling the mosquito population and better preventative matters. Mosquitoes breed in areas with standing water (such as old tires and flower pots). Removing objects that harbor stagnant water will help control the mosquito population. The *Aedes aegypti* mosquito usually bites during the day. Wearing long sleeves and pants when out doors and screening is a good preventative as well. A combination of prevention and vaccination would be an ideal way of better controlling DF.

Environmental control such as reducing vector breeding sites, proper solid waste management, improvement in substandard house design, and public education programs have been implemented in dengue control programs in an effort to reduce the number of dengue cases occurring in the population. The application of insecticides around dwellings is also a method used for eliminating the number of possible breeding sites for

mosquito. Active monitoring and surveillance of mosquito population and densities should also be implemented in dengue infection control programs in an effort to prevent man-vector combat.

Since there is a need to develop a vaccine that raises protective immune responses against all dengue serotypes, the vaccine development for dengue disease has been difficult. Vaccine protection against only one or two dengue viruses is not effective as it could increase the risk of more serious infection later on. The significant problems of multiple serotypes, antibody-dependant enhancement, (ADE), cross-reactive antibodies, sub-neutralizing levels of antibody, cross-reactive (low affinity) T cells, and problems in vaccines distribution have all been contributing factors in complicating the development and manufacture of an effective dengue vaccine (Fink *et al.*, 2006). Although many of the vaccines developed so far (live attenuated, chimeric DNA and subunit vaccines) show promising results, none have been proven to sufficiently immunogenic for routine use (Malavige *et al.*, 2004).

Disease Mechanisms

Dengue is transmitted by the bites of infected mosquitoes of the genus *Aedes*, most commonly *Aedes aegypti* (Fink *et al.*, 2006). Mosquitoes generally acquire the virus while feeding on the blood of the infected person. After biting the infected human, the virus enters the adult female mosquito where it first replicates in the midgut. From the midgut, the viral particles can be found within the nervous system, salivary glands, foregut, fat body, epidermal cells, ovary and internal body wall linings cells of the mosquito (Malavige *et al.*, 2004). Infected female mosquitoes are also capable of spreading the virus to their progeny, which allow it to act as reservoir for virus maintenance. After the virus incubation in the insect for a few days, the infected mosquito is then capable of transmitting virus through blood feeding exchanges with susceptible individuals.

The initial bites of the mosquito in the skin allows the virus to permeate epithelial layers in the skin, where virus first interacts with the resident dendritic cells. These cells are more permissive to dengue infection than monocytes or macrophages. Upon infection, dendritic cells express dendritic cell specific ICAM3-grabbing non-integrin receptor, a mannose specific C-type lectin, which binds to all four serotypes of dengue envelope glycoprotein (Fink *et al.*, 2006). Each serotype of dengue virus is different from one another. Recovery from infection of one serotype only provide lifelong immunity against that specific serotype but confers only partial protection from subsequent infection by the other three serotypes. The phenomenon known as antibody-dependent enhancement (ADE) exists in dengue epidemic where pre-existing antibodies against a primary infection cross-react with the secondary infection virus and enhances infection from other serotypes (Fink *et al.* 2006). The mechanism of ADE involves formation of complexes of dengue virus and pre-existing, non-neutralising antibody against a different dengue serotype. These complexes then bind via the Fc portion of the antibody to Fc γ RI and Fc γ RII bearing cells, which take up the complex, allowing the virus to escape and infect the cell (Fink *et al.*, 2006).

Dengue virus infection produces a spectrum of clinical illness ranging from a nonspecific viral syndrome to severe and fatal hemorrhagic disease. Majority of dengue virus infections are asymptomatic. However, it could also give rise to severe disease such as dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) which are life-threatening complications. Dengue fever is a self-limited febrile illness where a sudden onset of fever occurred after the incubation period of 2-7 days (Kurane and Takasaki, 2001). The fever is usually accompanied by other type of symptoms such as severe headache in retro-orbital area, arthralgia, myalgia, anorexia, abdominal discomfort and macular popular rash (Malavige *et al.*, 2004). However, patients usually recover from the symptoms without complications in several days following the onset of fever. Some patients infected with dengue virus can experience sudden leakage of plasma into interstitial spaces, which develops thrombocytopenia and possible hemorrhagic manifestation. This severe life-threatening syndrome is called DHF (Kurane and Takasaki, 2001). The World Health Organization (WHO) categories DHF into four grades ranging from grade one to grade four. Plasma leakage is most profound in grade 3 and 4 stage where the shocks occurred, known as DSS (Kurane and Takasaki, 2001). DSS is associated with high mortality.

Pathogen Structure

Dengue is a single stranded RNA virus that belongs to the family of *Flaviviridae*. It has four distinct serotypes within the flavivirus genus (DEN-1, DEN-2, DEN-3 AND DEN-4). Its genome consists of a single open reading frame directing the synthesis of a polypeptide, which is cleaved by viral and host proteases into ten viral proteins. Dengue virus proteins include three structural proteins such as core, envelope and membrane precursor proteins, in addition to several non-structural proteins. The envelope protein is involved in the biological functions of the virus as it binds to receptors on the host cells allowing the virus to be transported across host cell membranes (Malavige *et al.*, 2004). During the course of infection, dengue virus triggers a conformational change in its envelope protein, which permits the fusion of the virus envelope to the endosomal membrane, causing the release of the viral genome into the cytoplasm of the newly infected host cell. The virus then forms replication complexes on the membrane of the host cell's endoplasmic reticulum and begins its replication process. Immature virus particles are transported by the secretory pathway to the cell wall before they are secreted by the cell as mature virus (Fink *et al.*, 2006).

Description of Vaccine

A live-attenuated vaccine modifies the “wild” or pathogenic virus, allowing it replicate and produce immunity, while removing key virulence factors, limiting the risk of disease. Live attenuated vaccines induce stronger immunity opposed to killed vaccines, as killed vaccines do not stimulate the production of proteins in the host cytosol. Intracellular replication of a live attenuated virus enables antigen to be processed and presented by MHC I, effectively inducing CD8+ T cells and an overall cellular response. The risk of using a live attenuated vaccine can lead to many mutations in the genes encoding the

protein, making it possible for the pathogenic virus strains to reemerge by further mutation during viral replication. Although the attenuated strains replicate poorly in humans and do not allow the disease to develop, immune response to the attenuated vaccine is strong enough not to warrant the use of an adjuvant, and has been shown not to have little effect on antibody titer (Gamble, *et al.*, 1985).

We propose the development of a live-attenuated virus vaccine to combat Dengue virus. Immunity to all four serotypes is essential, as infection can be caused by exposure to any one serotype. Immunodominance becomes an issue when dealing with DF and DHF because of these four different serotypes. Previous exposure to any serotype makes individuals more susceptible for a more severe infection at a later exposure because the immune system responds less favorably. An individual may be exposed to all four serotypes of the virus and generate a response; however, the immune system will only respond to the serotype of the virus that generates the highest affinity T cells. In this way, this only allows protection against the serotype that caused initial infection.

Since during infection, lymph nodes are sites of antigen representation, a better immune response might be possible if vaccination for all four serotypes occurred simultaneously in different areas of the body. This would allow four different lymph nodes to help develop immune responses to all four serotypes and help eliminate immunodominance (Deem, 2004).

In order for the vaccine to work effectively, it must be able to generate a long-lived immunological memory because booster vaccinations are impracticable to give in these rural areas. The vaccine must be able to initiate a cell mediated immune response by developing memory T cells, as well as a humoral immune response by producing specific antibodies towards all Dengue serotypes.

Mode of Transmittance

To stimulate a stronger immune response from a dengue vaccine, vaccinating in four different areas of the body would be ideal. The problem with this is that many people may not allow for multiple injections, especially simultaneous injections. A different approach would be a transdermal vaccination, which would optimize an immune response using SonoPrep. SonoPrep is a device that permeates the skin's outer layer through low frequency ultrasound, which allows molecules to pass through much faster and more efficiently than regular vaccine administration (Tezel *et al.*, 2005). The problem with both an injection vaccine and the SonoPrep, is cost of administration. Most areas that are affected by DF and DHF are rural and not nearby medical facilities. In addition, many of these communities would not have money to pay for the vaccine. The cost to give four vaccines to one person can add up and become an issue in trying to resolve this endemic disease. Even if the major of the population is vaccinated against DF/DHF, those not vaccinated will still be protected from infection.

Vaccine Mechanism and original antigenic sin:

In the production of a dengue virus vaccine, stimulation of cellular immunity, especially that of cytotoxic T-cells, is proven necessary in irradiation of the dengue virus from an infected host. There is, however, controversy over the immunopathogenesis in the progression of DHF from DH (Kurane and Tomohiko, 2001). It is proven that while CD8⁺ T-cells aid in the removal of dengue virus, they also can become cross reactive for other dengue virus serotypes with a lower binding affinity (Fink *et al.*, 2006).

The complete mode of immunopathogenesis of dengue fever is still unknown, however, it is established through the use of immunocompromised mice (An J, *et al.*, 2004), that CD 8⁺ T-cell proliferation may lead to both removal of certain dengue virus serotypes, while weakly protecting against others. This established, secondary dengue virus infections introduce the possibility of CD 8⁺ T-Cell stimulated immunopathogenesis through weak binding affinity of CD 8⁺ T cells on MHC I receptors. Known as 'original antigenic sin', weak affinity memory T cell response poses a risk to the ability of the host immune system to make a strong response to multiple dengue virus infections. This is due to the way in which memory T cells respond to an infection. Memory T cells are usually advantageous to the immune system, as known pathogens create a faster immune response due to fast proliferation of memory T cells to produce antigen-specific CD 4⁺ and CD8⁺ T cells. In the case of Dengue fever, these memory T cells would only be affective if the same dengue virus serotype were to re-infect a host. In many cases, a secondary dengue infection is due to a different serotype, thus causing the immune system to respond with memory T cells specific for a different serotype of dengue virus (Fink *et al.*, 2006).

Cross reactivity associated with dengue fever is not limited to CD8⁺ t cells and CD 4+, but also non-neutralizing antibodies. As the infection stimulates production of IgG specific antibodies, binding of dengue virus to the Fcc receptors on host cells through Fc regions on IgG antibodies perpetuates enhancement of the initial infection (Kurane and Takasaki, 2001).

Since a single serotype specific vaccine would not be affective in immunizing against all serotypes of dengue fever, immunity for all four serotypes would be needed for maximum immunity against dengue virus (Raviprakash *et al.*, 2006).

This would prevent DH from occurring in people that had not been infected with Dengue virus previous to vaccination, and prevent DH from progressing into DHF by eliminating the possibility of a second infection in people that were vaccinated after a first infection. As dengue virus is comprised of four virus serotypes, a tetravalent live attenuated vaccine type would be proposed, as a live attenuated virus will induce both strong cellular immunity to all four pathogenic serotypes. Since there is cross reactivity among all serotypes, a tetravalent vaccine would prove difficult to manufacture, as each serotype would need to generate an immune response in order for immunity to be effective against the possibility of DHF (Raviprakash *et al.*, 2006)

DEN 3 serotype has been shown to elicit milder symptoms of Dengue fever, with less susceptibility to DHF, suggesting it is a naturally occurring partially attenuated virus. The risks to patients of using the structural proteins for this serotype are lower than that

of other serotypes if the attenuated virus was to revert to its virulent form. Due to this, DEN 3 structural proteins will be used in creating a chimeric vaccine (Blaney, *et al.*, 2004).

Using reverse transcriptase polymerase chain reaction (RT_PCR), cDNA clones of ME genetic regions from DEN1, DEN2 and DEN4 will be used and incorporated in to DEN3 (Porter, *et al.*, 2005). A cDNA site from DEN1 similar to that of DEN4 will be used to replace a section of ME coding region on DEN4. DEN4 chimera will similarly replace ME gene regions with that of DEN2 and incorporated into DEN3, creating a fully chimeric vaccine.

In having ME coding regions of all serotypes incorporated into the genome of DEN3 virus serotype, immunity against all serotypes can be made simultaneously. As the immunopathogenesis is still unknown, the precise sites of antigen recognition are unknown, however, it is well established that ME regions generate sufficient antibody and T_c response when tested on rhesus macaques (Blaney *et al.*, 2004).

Results:

By introducing a chimeric tetravalent live, attenuated dengue virus into an uncompromised host, it has been shown that in addition to creating specific anti-dengue IgG antibodies, immunized patients do not create high levels of TNF-alpha during subsequent infections (Fink *et al.*, 2006). This is of great significance, as high levels of TNF-alpha and other chemokines are known as contributing factors to DHF. This is likely attributed to reduced cross-reactivity among CD8⁺ and CD4⁺ T cells, as high levels of T cell activation produce a rapid cascade of chemokines over a extremely short time period, eventually leading to malfunction of epithelial cells, causing plasma leakage. Immunized patients with no previous dengue infection, and patients immunized after initial infections would require follow-up antibody testing to insure immunization against all dengue virus serotypes (Raviprakash, 2006).

The criteria for successful trial would be to show significant resistance to dengue infection over the lifetime of the patient after being vaccinated. To assess the effectiveness of the vaccine proposed, patients who were given the vaccine would be monitored against those who were given a placebo in order to document the rate of dengue infection among the two study groups. The patient serum level would be monitored every 3 months. Lower rate of dengue infection among participants who were being vaccinated would illustrate the effectiveness and success of the vaccine. As four simultaneous injections would be costly, the proposed route of administration for the tetravalent live vaccine would be through intravenous injection (Blaney, *et al.*, 2004).

Description of Immunity Assessment:

The immune response to dengue virus includes the production of IgM antibodies by the fifth day of primary infection. IgM antibodies remain in the circulatory system for thirty to sixty days, while IgG antibodies appear by the fourteenth day of infection and persist for life. A secondary infection, on the other hand, induces IgM antibody response after twenty days of infection and the rise of IgG antibodies within one to two days

following the onset of illness. The use of sensitive serological testing can help detect the presence of the anti-dengue IgG or IgM antibodies and provide serological diagnosis of the dengue infection. The enzyme-linked immunosorbent assay (ELISA) will be used to quantify the amount of specific antibody in the blood. First, the purified dengue virus is bound to the micro-well of the plate. To quantify antibody to the dengue virus, human serum will be added to the plate and incubated. If the antibody to dengue virus is present in the serum, the antibody will bind to the virus on the wall of the plate. To detect IgG or IgM antibodies to the dengue virus, the anti-human IgG or IgM antibodies are added to the well. If antibodies have been bound to the virus on the wells, the enzyme conjugate will then bind to the antibodies. After another series of washes, a chromogen is added to the well. If the enzyme conjugate is present, peroxidase will catalyze a reaction that consumes the peroxide and turn the chromogen from clear to blue in color. The intensity of the color correlates to the amount of enzyme present on the well. The amount of antibody in the unknown specimen can be determined by using the standards with known amount of antibody and comparing the intensity of the color produced by the unknown serum specimen to the standards. If the antibody to dengue is IgG, the anti-human IgG antibody added would bind to the human IgG in the well. The amount of enzyme-labeled antibody bound will depend on the amount of antibody bound to the virus. IgG is the most prevalent immunoglobulin in serum and is found mostly in tissue fluids and lymph. IgG immunoglobulins are responsible for labeling the targets for removal by phagocytes. Since both macrophages and neutrophils have receptors for the Fc region of IgG, they could bind to and engulf the dengue virus coated with antibody (Lam and Devine, 1997).

ELISPOT Assessment:

To study the T cell response to peptide antigens of dengue, enzyme-linked immunosorbent spot (ELISPOT) test will be conducted. Peripheral blood mononuclear cells (PMBC) from the blood will be first obtained from patients experiencing secondary dengue virus infections. Collected PMBC samples will then be tested using IFN- γ and TNF- α ELISPOT assays. Polyvinylidene difluoride (PVDF)-backed microplates will be precoated with anti-IFN- γ or anti-TNF- α monoclonal antibodies. Antigen peptides and isolated PMBC will then be plated out in varying densities for overnight incubation at 37°C in a CO₂ incubator. Cells and media components will then be removed by washing the plates with PBS before addition of biotinylated IFN- γ or TNF- α detection antibody to detect the specific captured cytokine. Following a wash to remove any unbound biotinylated antibody, the alkaline phosphatase conjugate substrate will be added to visualize spots formed by IFN- γ or TNF- α secreting cell. In each assay, mitogen leukoagglutinin will be used as positive control, while irrelevant peptides of dengue would be used as negative control. Visualizing spots will be counted via dissecting microscope.

ELISPOT will be used to measure the T-cell response because of its ability to detect components of cellular immunity. The cytokines IFN- γ and TNF- α are measured in ELISPOT because of their ability to induce plasma leakage in patients with DHF.

Flow Cytometry:

Flow cytometry is becoming a more widely used technique in determining the distribution of T cells by marking different cellular structures with the use of fluorescent dyes and measuring the fluorescent light emissions. Both CD8⁺ and CD4⁺ T cells use TCR to bind peptide on MHC class I and II, respectively. Tetramers of MHC-peptide bind to peptide-binding T cells which are detected with the use of flow cytometry. Fluorochrome-tagged monoclonal antibody directed against CD8⁺ and CD4⁺ T cells is added to the sample. Some fluorochromes used are fluorescein, phycoerythrin (PE), allophycocyanine (APC), and Texas red. Once the fluorochromes are added, the cells are incubated and run through the flow cytometer. As the cells pass through the laser beam, the fluorochrome(s) used become “excited”, and the amount of light scattered, or absorbed and reemitted is analyzed. A fluorescence-activated cell sorter (FACS) counts the number of cells with specific characteristics and measures the levels of expression of molecules on these cells. The number of cells identified can determine whether the vaccine initiated good cellular immunity.

The development of memory cells and CD8⁺ T cells against a viral infection, like Dengue, is dependent upon CD4⁺ T cells. CD4⁺ T cells produce Th1 cytokines, IFN-gamma and TNF-alpha, which stimulate an antiviral state in the host and help initiate cellular immunity during a viral infection. A study done by the Center for Infectious Disease and Vaccine Research at the University of Massachusetts Medical School used peripheral blood from six volunteers who had previously received experimental live, attenuated Dengue vaccines to measure the amount of CD4⁺ T cells with the use of flow cytometry. Intracellular cytokines and T cell activation markers were detected using PE-Cy5-conjugated anti-CD69, FITC-conjugated anti IFN-gamma, and allophycocyanin-conjugated anti-TNF-alpha mAbs (BD Pharmingen). After analyzing the numbers, cytokine-positive CD4⁺ T cells were found in the volunteers’ peripheral blood samples. This may indicate that a vaccination against Dengue could initiate good cellular immunity. (Mangada and Rothman, 2005)

Discussion:

In principle, an effective vaccine against dengue virus seems highly feasible since it causes only acute infection and the virus replication could be effectively controlled after a short period of time. However, there are many problems in development of an effective vaccine. One of the problems is the lack of understanding in the pathogenesis of DHF, and absence of sufficient animal testing for the dengue disease. Since the effective vaccine has to be tetravalent and prevent infection from all four serotypes simultaneously, it poses many challenges in the manufacturing of such a vaccine. In past attempts, tetravalent formulations that retain immunogenicity to all four serotypes has been difficult, as it requires the use of complicated, multi-dose immunization regimens (Chaturvedi *et al*, 2005). One of the significant obstacles in development of effective vaccines for dengue virus is the inability to predict effectiveness of the vaccines in preventing DHF. This is because the vaccine could only be analyzed in experimental

animal models, which do not reproduce the same DHF syndrome seen in humans (Chaturvedi *et al*, 2005).

Upon further testing on animals with comparable immune responses to humans against dengue virus, such as the Rhesus Monkey, (Blaney *et al.*, 2006) and further research into the exact immunopathogenicity of the virus, it is proposed that such live, attenuated vaccines will be an affective means to immunize high risk regions against dengue virus, effectively eradicating a major worldwide heath problem.

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