Trop. Med., 35 (4), 233–241, December, 1993

Strategies of Dengue Vaccine Development by W. H. O. Using New Biotechnology

Kotaro YASUI

Department of Microbiology and Immunology, Tokyo Metropolitan Institute for Neuroscience, 2-6, Musashidai, Fuchu, Tokyo 183, Japan

Abstract: Dengue fever and dengue haemorrhagic fever (DHF) with shock syndrome are found in tropical and subtropical areas of Asia, Africa, Western Pacific and Central and South America. DHF is one of the major causes of hospitalization of children in urban of these areas. Control of the mosquito is the only means available now to curb the disease. Therefore a vaccine is urgently needed. Sequential infections with different dengue virus serotypes represent a risk factor for DHF. Prior immunity to a heterologus serotype enhance virus infection of another serotype in macrophages and monocytes. Thus, the approach of the Steering Committee (SC) of W. H. O. is to develop a tetravalent candidate vaccine including 4 serotype antigens or to engineer dengue vaccines without the component of immune-mediated enhancement.

The strategic plan of the SC had included (a) definition of protective epitopes; (b) expression of protective epitopes; (c) definition of virulence in molecular terms. In view of the fruits, the following strategic plans of the SC were included. i. Infectious clone research using parental and candidate vaccine strains and definition of virulence and attenuation in molecular terms. ii. Construction and expression of protective immunogens by genetic engineering techniques. iii. Development of animal model for DHF. iv. Development of candidate vaccines.

Flaviviruses and their vectors are now spread over a very large area of the world. Dengue is a mosquito-borne disease which occurs in almost all tropical countries including some subtropical areas. Dengue is the most serious and rapidly rising arbovirus infections in the world. The best estimation is that millions of DHF cases occur each year, with thousands of deaths, but this is probably an underestimate. The number of dengue epidemics has substantially increased in the past 10 years. The table 1 shows countries or territories in which dengue or DHF is known to occur, in 1975-1993 by W. H. O. One fifth of the world's population is at risk of dengue in these areas. Dengue now causes massive epidemics in non-immune population and income loss incurred during dengue and DHF epidemics is a major economic problem in these countries. The recent increase in dengue transmission has resulted mainly from rapid urbanization in endemic countries. Although

Table 1. Countries or territories in which dengue or dengue haemorrhagic fever is known to occur, by W. H. O. region, 1975-1993

The Americas (40)	Antigua, Aruba, Bahamas, Barbados, Belize, Bolivia, Bonaire, Brazil, British Virgin Islands, Curaqao, Colombia, Cuba, Dominica, Dominican Republic, Ecuador, El Salvador, French Guiana, Grenada, Guadeloupe, Guatemala, Guyana, Haiti, Honduras, Jamaica, Martinique, Mexico, Montserrat, Nicaragua, Paraguay, Puerto Rico, Saint Kitts and Nevis, Saint Lucia, Saint Martin, Saint Vincent and the Grenadines, Suriname, Trinidad and Tobago, Turks and Caicos Islands, U. S. A., Venezuela, Virgin Islands of the U. S.,
South-East Asia (7)	Bangladesh, India, Indonesia, Maldives, Myanmar, Sri Lanka, Thailand,
Western Pacific (26)	American Samoa, Australia, Cambodia, China, Cook Islands, Fiji, French Polynesia, Guam, Kiribati, Lao People's Democratic Republic, Malaysia, Marshall Islands, Nauru, New Caledonia, New Zealand, Niue, Palau, Philippines, Samoa, Singapore, Tokelau, Tonga, Tuvalu, Vanuatu, Viet Nam, Wallis and Futuna Islands,
Africa (18)	Burkina Faso, Comoros, Cote d'Ivoire, Ethiopia, Ghana, Guinea, Kenya, Madagascar, Mozambique, Nigeria, Senegal, Seychelles, Sierra Leone, Somalia, South Africa, Sudan, United Republic of Tanzania, Zaire,
Europe (1)	Bosnia and Herzegovina,
Eastern Mediterranean (0)	

all ages suffer from dengue fever, DHF is most commonly observed in children. During severe epidemics many of the infected infants and children can die, even if properly treated. Control of the mosquito is the only means available now to curb the disease. Therefore a vaccine is urgently needed.

The problem

Dengue virus is classified into 4 distinct serotypes, named dengue 1, 2, 3 and 4, and any of the four serotype viruses cause classic dengue fever in humans. No cross-protection exists among these serotypes, and an individual who has recovered from one serotype of dengue infection and developed antibodies against the virus remains susceptible to infections by other dengue viruses. While it is generally a debilitating but non-fatal illness so called classical dengue fever, a proportion of infected patients experience a more severe form of the disease called DHF which is often complicated by shock. Unfortunately, a second infection with a different serotype of dengue virus has been associated with an enhanced risk of developing DHF and death(Table 2). For this reason, dengue vaccines cannot be fielded one at a time as they become available: it appears essential that an

immunization program against dengue should provide simultaneous protection against all four serotypes. The precise mechanism of developing DHF is not clear and an efficacy test for a candidate vaccine cannot be done on DHF now, because an animal model for DHF is still lacking, however several hypothesis have been postulated.

The immune enhancing or antibody dependent enhancing phenomena of virus infections have been observed in vitro not only on dengue virus but also on other viruses. Dengue virus can infect adherent human monocytes. When the dengue virus is mixed and formed immune complexes with cross-reactive antibodies, infectivity of the dengue virus is greatly enhanced. The concept that dengue viruses can infect human monocytes more easily as an immune complex with non-neutralizing antibodies via a Fc receptor on the cell surface for immunoglobulines than as individual virion via a cellular receptor for dengue virus have been accepted to explain the antibody dependent enhancement of dengue virus infectivity in vitro (Table 3). Similar phenomena were also observed on other virus infection. On Japanese encephalitis virus (JEV) infection, pre-existence of certain non-neutralizing monoclonal antibodies against JEV induced earlier death on the mice infected

Table 2. Dengue virus and its disease

Dengue virus	type 1
	type 2
	type 3
	type 4
Classical dengue fever	
Dengue haemorrhagic fever (DHF)	
Dengue shock syndrome (DSS)	
DHF/DSS occurs at relatively high frequency in infants and children	
DHF/DSS occurs at relatively high frequency during secondary dengue infections	

Table 3.

Antibody-dependent enhancement of dengue virus infectivity
Dengue virus can infect human monocyte more easily as an immune complex with non-neutralizing antibodies via a Fc receptor for immunoglobulin than as an individual virion via a cellular receptor for dengue virus

with JEV. As shown figure 1, mice received non-neutralizing monoclonal antibody 201 or 302 passively 1 day before JEV inoculation died earlier than mice received normal mouse IgG, however mice received neutralizing antibody 503 were completely protected from JEV infection (Figure 1). It is not clear whether antibody dependent enhancement of dengue virus infectivity is first step for development of DHF in vivo. However, because DHF and dengue shock syndrome (DSS) occurs at relatively high frequency during secondary dengue infections and in infants less than 1 year old, a candidate vaccine and its immunization program for dengue should induce simultaneous good protection against all four serotype viruses.

Targeted research activities and strategic plan

JEV is a close biophysical relative of the dengue viruses and can cause severe disease throughout much of Asia. At least 40,000 cases of JE is estimated in each year, of which one third will die and one third will suffer very serious long-term effects. Although inactivated vaccine made from homogenized, infected mouse brain or using primary hamster kidney cells is used by a number of countries, an inexpensive, single inoculation vaccine that induces long-lasting immunity is needed. So W. H. O. also support the development of new JEV vaccine using new biotechnology as same as dengue vaccine.

The development of inactivated dengue vaccines has not been previously achieved because these viruses do not grow to high enough yields in cell culture to produce sufficient

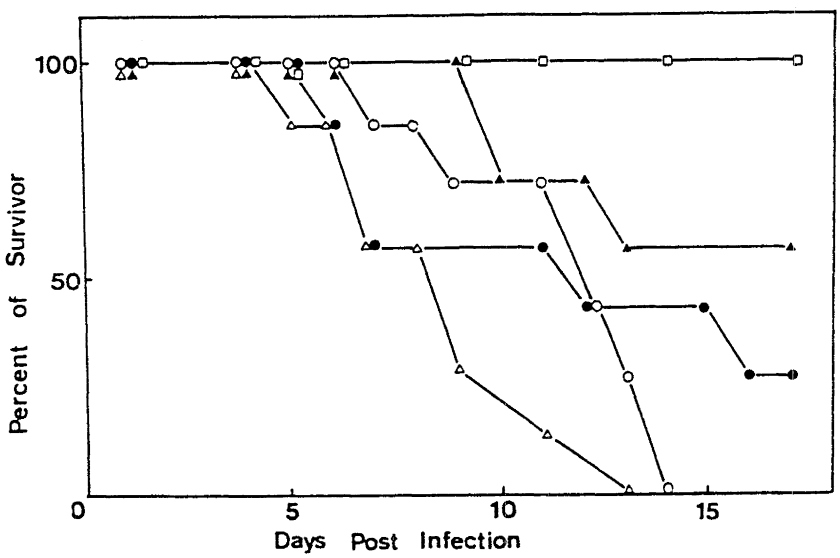

Fig. 1. Earlier death of JEV-infected mice by passive administration with certain mAb. The mice were administered i. p. with 200 μg/mouse of each mAb 1 day before JEV inoculation. Symbols: ○, control (normal mouse IgG): ●, mAb 201; △, mAb 302; ▲, mAb 109; □, mAb 503; Similar results were obtained from two or more independent observations.

antigen for successful immunization. After many efforts, live attenuated candidate vaccines have been developed at Mahidol University, Thailand and at the Walter Reed Army Institute of Research, U. S. A. The tetravalent dengue candidate vaccines developed at Mahidol University are now tried on phase 1 and 2 test in Thailand with support of W. H. O./SEARO, as reported by Dr. Natth Bahamarapravati, but second generation vaccine will also be needed.

The strategic plan of the Steering Committee on Dengue and Japanese Encephalitis Vaccine, elaborated in 1987, is shown in Table 4. The main aims is to produce second generation vaccines by using these approaches and its time frame is shown in Figure 2. The initial aims have been accomplished to a large extent on several parts. Protective

Table 4.

(a) Identification of critical (protective, neutralizing and enhancing) epitopes.

(1) Human sera targets (viral protein exposure)

(2) Mouse monoclonal antibody production and characterization

(3) Active mouse immunization (with isolated viral proteins)

(4) Passive mouse immunization (with monoclonal antibodies)

(b) Expression of critical (protective/neutralizing) epitopes.

(1) cDNA cloning and sequencing

(2) Expression and/or synthesis of viral proteins and/or peptides

(3) Immunization experiments

(4) Challenge experiments

(c) Optimization of immunization.

(1) Adjuvants/immuno-enhancers

(2) Expression systems (*E. coli*, yeast etc.)

(3) Self-replicating vectors (yellow fever, vaccinia etc.)

(d) Molecular definition of virulence.

(1) Cloning, sequencing and comparison of parent versus vaccine strain pairs

(2) Cloning, sequencing and comparison of "classical" dengue versus dengue haemorrhagic fever strain pairs

(e) Construction of engineered live attenuated vaccines.

(f) Similar lines of research will be supported on JE.

epitopes on E proteins were identified. NS1 protein was also shown to confer protection, although not inducing neutralizing antibodies. Expression of the E and other proteins was successfully accomplished in *E. coli*, vaccinia virus and baculovirus systems and other expression systems of the E protein in mammalian cells have been developed. The genetic basis for virulence was studied by examining sequences in parent and candidate live attenuated vaccine pairs. Infectious clones of yellow fever, dengue 4 and JEV have now been made. Infectious clones and chimeric viruses may be one of possible candidate vaccines. Purified subunit truncated E protein is reportedly immunogenic and can be produced in baculovirus systems(Table 5). Recombinant vaccinia viruses, baculoviruses, and other expression vectors produce small immunogenic particles including E and M proteins of JEV and these particles elicit neutralizing antibodies and protect mice from the challenge by JEV and prevent the viremia in pigs. Studies with vaccinia recombinant viruses in human subjects are in the planning stage. Briefly, when preM and E coding region genes are exressed by vaccinia virus, baculovirus or mammalian cell expression systems, E and preM proteins are produced in cells as E-preM heterodimer. And these proteins are released from the cells to culture supernatant as about a 20 nm envelope particle containing E and processed M proteins as E-M associating complex. The E-M associating complex expresses biologically active epitopes well, but E alone does not. And E-M envelope particle can elicit protective immunity in animals excellently(Table 6). These appoach is now being extended for dengue viruses. From these progress, the SC

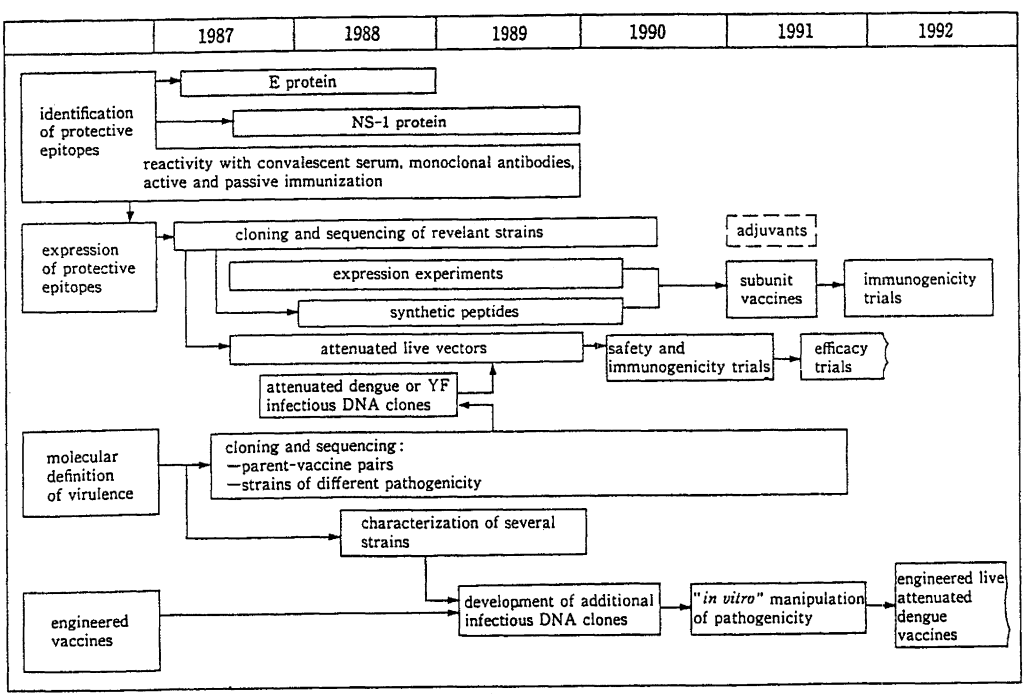

Fig. 2. WHO strategy to develop the Second generation dengue vaccine

modified the priorities of research for development of the second generation vaccine as shown in Table 7.

The SC recommended following project for funding 1993.

Renewal projects

BLAIR (USA): Identification of dengue 1 virus genome sequences that mediate antiboby -enhancement of dengue-2 viruses.

Table 5. Vaccine approach

Infectious clone
YFV, JEV, D-4,
Live vector
vaccinia, rubella, adeno, Semliki Forest, Salmonella, BCG,
Subunit including NS antigens
baculo, peptide, purified proteins, bacteria cells,
Particle
vaccinia, baculo, mammalian cells, liposome, ISCOM,
Immunology
epitope mapping, structure of the antigens, T cell, ADE, Receptor, targer (Dendric cell) cells,

Table 6. Expression of flavivirus E envelope particles and development of protective immunity in animals with the particles

ss preM-E coding region of flavivirus genome
vaccinia virus, baculovirus, and other animal cell expression systems
expressed extracellular E envelope particles or cell surface E
good expression of biologically active epitopes and excellent immunogens to elicit protective immunity in animals.
ca 20nm particles which contain E and M protein.
chimera particles with different flavivirus E proteins are available.

Table 7. Research priority of WHO dengue vaccine development

(a) Infectious clone studies using existing parental and candidate vaccine strains. These studies will include elucidation of the molecular definition of virulence and attenuation.
(b) Studies which utilize available data on protective antigenic sites to construct and express protective immunogens. Approaches envisioned would include recombinant DNA generated particles or antigens vectored by currently available delivery methods, and chimeric particles or viruses.
(c) Research evaluating, comparing, and then optimizing the protective efficacy of these antigens in current or newly developed animal model systems.
(d) Projects aimed at the development of vaccine candidates including methodologic research on production, purification and process development, and manufacture of pilot lots under Good Manufacturing Practice (GMP) guidelines.

GALLER (Brazil): Molecular analysis of yellow fever vaccine viruses
SHOPE (USA): Production and evaluation of vaccinia vectored dengue virus vaccines
SHOPE (USA): Engineering an attenuated strain of Japanese encephalitis virus for use as a human vaccine
STRAUSS (USA): Expression of flavivirus proteins

New projects
DEUBEL (France): Expression, purification and evaluation of the protective efficacy of the four serotypes of dengue E protein fused to MalE
SUMIYOSHI (USA): Development of an infectious cDNA clone for dengue type 3 virus
YOUNG (Australia): Baculovirus expressed immunogenic particles as candidate sub-unit dengue virus vaccine
BRINTON (USA): Molecular analysis of dengue virus-specific, human cytotxic T-cell immunodominant epitopes
COMPANS (USA): Virus-like particles as dengue vaccine

The 1993 World Health Assembly in a resolution has noted the worldwide importance of dengue haemorrhagic fever as an increasing disease problem. The resolution called for vaccine development in addition to better diagnostic and control of the vectors. Because there are severe restrictions in funding of W. H. O. for coming year clear programme focus

is essential in order to achieve the SC goal of efficacious, safe, inexpensive vaccine for dengue and JEV in the near future.

This paper was written according to W. H. O. SC reports.