



ARBOVAX®

ADVANCED TECHNOLOGY FOR INSECT-BORNE VIRUS VACCINES

Arbovax Dengue Virus Serotype 2 Vaccines Produce Strong, Protective, Neutralizing Antibody Response in African Green Monkeys

Dengue disease, typically recognized as dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS), is caused by four closely related but antigenically distinct serotypes (DV1-4). Dengue virus is a member of the flavivirus family and is spread by mosquitoes, most commonly in tropical and sub-tropical environments. Although often asymptomatic, in 5-10% of clinical cases, particularly in children, dengue infection results in DHF and DSS. Morbidity, mortality and overall economic impact associated with DHF and DSS remain high. Protection against homotypic reinfection is complete and probably lifelong but no cross protection is afforded to the different serotypes following infection by any one of the individual serotypes. More important is the fact that the presence of antibodies to one serotype of the Dengue virus facilitates the occurrence of Dengue Hemorrhagic Fever through “immune enhancement”(upon infection with a second serotype [1]. During immune enhancement, the virus/non-neutralizing antibody complexes are preferentially engulfed by cells of the immune system that then enable the virus to replicate unchecked. This scenario leads to increased viral load and more severe, often fatal, disease. For this reason, the development of a vaccine that produces a balanced immune response to all four serotypes is absolutely critical.

Arbovax Vaccine model and Innovations

Historically the use of a whole, infectious but attenuated virus is the most effective in producing a strong, lasting and protective immunity. Use of denatured virus, protein subunit or DNA vaccines to immunize does not present the host with antigen in its native conformation, which may lead to a weak immune response and no lasting immunity to challenge. For a successful vaccine, the immunogen should contain epitopes, or regions that bind antibody, which mimic the wild-type virus, so that any neutralizing antibodies made against the immunogen will recognize and counteract a naturally acquired viral infection. Most all these vaccines developed with the above alternate protocols will certainly require one or more booster doses to eventually confer full immunity. This paradigm is especially true for Alphaviruses and the similar-structured

Flaviviruses—such as Dengue virus, whose membrane-anchored glycoproteins are folded into compact, high-energy structures [2-5]. The folding process resulting in the production of the high energy, native configuration is complex, delicate and involves interactions with other virus proteins and molecular chaperones [2, 4]. This latter point suggests that vaccine strategies that employ expression of domains of these proteins as subunits or as components of other virus proteins may not produce the conformation of the protein that exists in the mature infectious virus, and thus will not allow production of antibodies that would neutralize the proteins of the wild-type virus (i.e. immunity to the native virus would not be conferred).

Of significant importance are neutralizing antibodies, which will bind and inactivate a viral invader and are a necessary component of a successful vaccine. However, other types of antibodies that bind but do not inactivate virus (non-neutralizing or sub-neutralizing antibodies) can also be generated by vaccines that do not use the intact virus, such as chimeras or sub-unit vaccines. In the case of Dengue Fever, virus exposure that leads to high levels of non-neutralizing antibodies is linked to occurrences of the more severe and often fatal Dengue Hemorrhagic Fever upon infection with subsequent Dengue virus serotypes [6]. In order to make a safe and effective vaccine against Dengue, the vaccine must protect from all four Dengue Virus serotypes while also initiating the desired immune response (neutralizing antibodies) and not generating the potentially deleterious non-neutralizing antibodies.

To overcome the problems associated with subunit and other vaccine designs resulting in the production of a safe and effective Dengue vaccine, Arbovax has exploited the fact that Arboviruses have evolved to replicate in the unique biochemical environments of both insect and mammalian hosts [7]. Since all viruses need to enter the cells of the host in order to effectively reproduce and cause disease, Arbovax explored unique and innovative methods by which this mechanism can be interrupted or limited in some way. Like all viruses, Arboviruses are not able to replicate without a host cell. Arboviruses do not make their own viral envelope but rather derive their encapsulating membrane from the host cell. Therefore, the Dengue virus produced in mosquitoes is different from the virus produced in mammals. Insect cell membranes do not contain cholesterol and are thinner in cross-section than mammalian cells [8]; therefore, the membrane-spanning domain of the virus—known as the transmembrane domain, essential for the

attachment and entry into the host cell—has different chemical and physical requirements to effectively enter and reproduce in insect and mammalian cells. This difference was utilized to develop a method for production of viral mutants with truncated transmembrane domains capable of efficient growth in insect cells but incapable of productive replication in mammalian cells [9, 10]. The altered transmembrane domains are embedded within the virus’s protective outer membrane so that all virus ectodomains—or outside surfaces that are recognized as foreign by the host’s immune system—are indistinguishable from those of the wild-type Dengue virus. By this method, the best possible conditions for a strong and long-lasting immune response are generated in the vaccine recipient. This method of modifying viruses produces “Host-Range Mutations,” so called since they are limited in their ability to reproduce in different species.

This strategy is the basis for the development of vaccines against insect-vector-borne, membrane-containing viruses like Dengue Fever. Introducing an attenuated virus deficient in infectivity gives the host immune system the chance to mount a defense before the onset of clinical symptoms. This will stimulate an immune response with a high concentration of neutralizing antibody to provide protection against the wild-type virus, without causing disease. In principle, this technology can produce a live-virus vaccine against any arthropod-vector-borne disease.

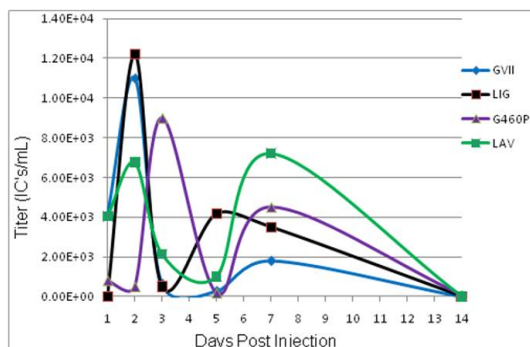
Arbovax’s vaccine strategy is based upon the straightforward concept of developing stable mutations of arboviruses that are capable of successful replication in insect cells but grow poorly in mammalian cells. This technique has the following advantages over traditional and recently introduced vaccine strategies:

- **Safety:** The modified virus is severely replication impaired in the patients’ cells, which allows sufficient time to develop immunity in the absence of disease.
- **Efficacy:** This strategy provides all epitopes of the virus to the host immune system and is immunologically indistinguishable from the wild-type (as compared to attenuated viruses, viral subunit proteins or viral nucleic acid anti-sense fragments).
- **Cost:** The cost of production will be significantly less than recombinant technology as the mutant virus can be easily grown in a standard insect-cell reactor. Initial U.S. estimates indicate that the manufacturing cost per dose for the final tetravalent product will be less than \$0.50/dose.

Initial proof-of-concept research was conducted with the Sindbis Virus. Sindbis is an Alphavirus that can produce fatal infection in mice. Two host-range mutants with deletions of 10 amino acids and 9 amino acids in the transmembrane domain of the E protein of Sindbis virus were created and characterized [9, 10]. These mutants produced virus that grew well in insect cells and poorly in mammalian cells. When tested for their ability to produce an immune response, both mutants induced antibody expression in vaccinated mice and one induced high titers of virus neutralizing antibody. The mutant with high neutralizing antibody titers also displayed 100% protection from mortality and morbidity, compared to 92% morbidity and 35% mortality in unvaccinated mice. This data proved the Arbovax concept: live, attenuated host-range viral mutants can be utilized as vaccines. For vaccine production, it will be necessary to screen a number of deletion mutants to find one with that produces the best neutralizing antibodies *in vivo*. The Sindbis virus deletion mutants were found to be stable in both cultured mosquito cells and adult mosquitoes. No reversions were seen after sequential passage *in vitro* or *in vivo* (Hernandez & Bowers, unpublished data). This finding is expected, since the host range mutant viruses replicate normally in the mosquito cells and are, therefore, under no selective pressure to revert to a wild-type form in this vector.

A strategy similar to Sindbis virus was applied to Dengue virus serotype 2. Over fifty mutants were created in transmembrane domains of dengue virus. After screening, three host range mutants were identified for further analysis. The viral mutants were stable in culture and capable of instigating a neutralizing antibody immune response in mice. A subsequent vaccine trial in African green monkeys resulted in strong neutralizing antibody production and protection from challenge with a virulent Dengue virus 2 strain by each vaccine candidate; all this was accomplished with *no boost*. After inoculation of animals with each of the three Arbovax DV2 vaccines plus controls, a live-attenuated DV2 virus (LAV) strain developed by the US Army and

Figure 1. Titers of DV2 vaccines two weeks post-inoculation.



mock (buffer injected), limited replication of each vaccine was established (Figure 1). In order to initiate a full immune response, some replication of virus is necessary. By 2 weeks post-inoculation, the animals have cleared each vaccine. In order to assess the

immune response of the monkeys to each vaccine, neutralizing antibody titers (Figure 2) and total IgG titers (Figure 3) were analyzed during the course of the study. Challenge with a wild-type virulent DV2 strain was performed on day 57.

Figure 2. Neutralizing antibodies generated in response to DV2 vaccines pre- and post-challenge.

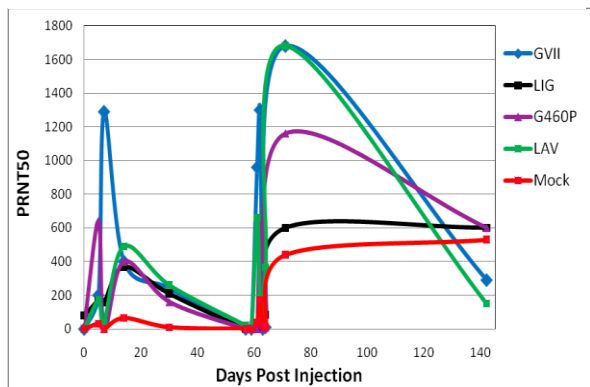
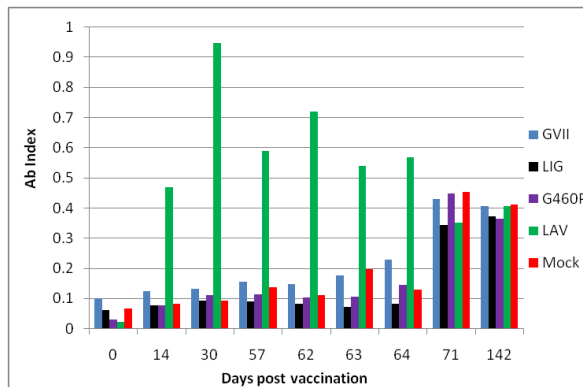


Figure 3. Total IgG Antibody pre- and post-challenge.



The three Arbovax vaccines each generated a strong neutralizing antibody response after initial inoculation and following viral challenge. Pre-challenge, the vaccine DV2ΔGVII showed the highest levels of neutralizing antibody (Figure 2). After viral challenge, levels of neutralizing antibody response were similar for DV2ΔGVII and the control vaccine LAV; however, neutralizing antibodies are a subset of the total IgG and in Figure 3, very high levels of IgG are observed for LAV. The Arbovax vaccines generate similar levels of neutralizing antibodies and total IgG, signifying that *very little non-neutralizing antibodies are generated by these vaccines*. However, LAV (Figure 3, green bars) generates much higher levels of IgG than neutralizing antibody. The result is a lot of non-neutralizing antibody production, which is deleterious for anti-dengue vaccine. Data strongly suggest that the Arbovax vaccines generate a predominantly neutralizing antibody response with very little detectable non- or sub-neutralizing antibodies. Currently Arbovax is working on a tetravalent Dengue virus vaccine formulation which would include host range mutants of each of the four Dengue virus serotypes.

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