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PERSPECTIVES FOR THE DEVELOPMENT OF DENGUE VIRUS VACCINES

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Introduction

The **Flaviviridae** is a family of about 60 viruses many of which cause diseases in man. Based on serological reactivities these viruses have been classified (De Madrid and Porterfield, 1974) in 3 main groups: yellow fever, dengue and encephalites. The encephalites group is further subdivided according to the insect vector (mosquitoes or ticks).

The mosquito-borne viruses yellow fever, dengue and encephalites account for millions of cases annually in tropical areas world-wide. Yellow fever is endemic in Africa (with a recent outbreak in Nigeria, De Cock et al, 1988) where transmission cycle is more complex than in the Amazonian Basin and Central western part of Brazil, both endemic areas (Pan American Health Organization, 1984). Here a few dozen cases/year are reported but this is clearly an underestimate being all classified as jungle yellow fever. The reinfestation of urban centers with the mosquito vector **Aedes aegypti** and the ease of transportation in and out of endemic areas have raised considerably the risk of reurbanization of yellow fever (see Coimbra et al, 1987). That this is possible is further corroborated by the recent outbreaks of dengue virus, transmitted by the same insect vector, in the last couple years in Brazil (Schatzmayr et al, 1986). The availability of a safe attenuated yellow fever virus vaccine and its large production in the country, together with vector control programme have so far avoided the return of urban outbreaks.

Dengue viruses constitute an ever-increasing health problem throughout the tropical areas world-wide. The four serologically distinguishible types account for the very high morbidity observed. All four serotypes are endemic in southeast pacific, the caribbean region and some countries in Central America with outbreaks of or more serotypes at a time. Dengue viruses have shown an amazing spreading tendency which is probably due to the reinfestation of many countries with the insect vector and/or the control of yellow fever virus. The outbreaks in Brazil are a

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good example for that and it seems to be a matter of time to have the other serotypes besides serotype 1 introduced into the country. Despite the fact that classic dengue is a relatively mild febrile state not always accompanied by headache, joint pain and rash, there is a major need vaccines arising from the existence and persistent spread of dengue hemorrhagic fever (DHF) or and dengue shock syndrome (DSS) with high morbidity and significant mortality (Monath, 1986). Currently two hypothesis would explain the occurrence of these severe forms of dengue; a) the virulence of the serotype and its strain (Rosen, 1986); b) the sequential infection, where preexisting antibodies of a primary infection in subneutralizing titers would enhance a secondary infection, specially if the latter virus belongs to serotypes 2 and 4 (Halstead, 1988). Dengue vaccines, therefore, must be effective and safe when used in these regions where DHF/DSS occur and where exposure to multiple serotypes is likely. To date, no reliable dengue vaccine, for any serotype, is available for mass vaccination, although some candidates have been obtained by serial passages in tissue culture.

Regarding the encephalites group, Japanese encephalites (JE) is by far the most serious case, given its high morbidity and in some instances mortality and/or permanent damage to parts of the nervous system. It accounts for several hundreds of thousands cases/year in Asia and its vector, ***Aedes albopictus***, is now present in the Americas as well. There is a killed JE vaccine which requires periodic immunization (Kitano et al, 1986).

All in all, it is clear that flaviviruses in general and specially dengue viruses are likely to grow as major health problem. The classical technique for obtaining attenuated viruses, i.e. serial passage in cell culture, although promising has met with little success for dengue viruses. Alternatively, there is hope that recombinant DNA technology might help elucidate viral replication mechanisms and possibly other biological parameters such as virulence/attenuation. This knowledge would be fundamental for designing second generation dengue vaccines.

This paper reviews (not exhaustively) the studies of several aspects of flavivirus genome structure and sequence variability; gene expression and epitope mapping and finally the synthesis of viral proteins in heterologous systems; as related to dengue vaccine development.

Virion morphology and composition

Flaviviruses are spherical viruses about 40-50 nm in diameter with an isometric, probably icosaedral, nucleocapsid surrounded by a lipoprotein envelope containing host cell lipid and two virus-specific polypeptides, the envelope (E) and the membrane (M) proteins. A capsid (C) protein complexes the nucleic acid forming the capsid. The virions contain single-stranded RNA (3-4x10E6 d) which is infectious.

Viral replication.

Flavivirus entry into cells follows the endocytic pathway and the proccess of virus replication and virus replication take place in the cytoplasm. Budding occurs into cytoplasmic vesicles which are then transported out of the cell. Upon uncoating the positive stranded RNA is translated in the rough endoplasmic reticulum giving rise to a unique polyprotein precursor of more than 3000 aminoacids which is then cleaved off to generate the structural and the nonstructural proteins (Cleaves, 1985).

In the flavivirus genome structural proteins are coded by the first quarter of the RNA with the remaining encoding the nonstructural proteins (NS1, ns2a/2b, NS3, ns4a/4b, NS5; Rice et al, 1985; Coia et al, 1988). The cleavage sites are rather well conserved among several flaviviruses not only on their position but also the aminoacid sequences as deduced from cDNA and protein sequencing (Rice et al, 1985; Bell et al, 1986; Biedrzycka and Wright, 1987; Hahn et al, 1988). The proteases involved in each of these cleavages are as yet unknown with the existing hypothesis based on the specific aminoacid sequence cleaved. Assignment of specific function to each of the nonstructural proteins is also incomplete. In fact, only NS3 and NS5 have been suggested as the RNA replicase components based on the high degree of aminoacid sequence conservation among flaviviruses. Isolation of replication complexes is technically difficult but plus and minus strands can be purified, also partially hybridized as shown by RNase resistance (Brinton, 1986). It is possible that host proteins are involved in each plus and minus strands synthesis initiation and elongation and this could be of importance regarding permissiveness and genetic resistance to flaviviruses.

The establishment of persistent infections have been associated with the generation of defective interfering particles (DIs; Holland et al, 1982) during RNA replication. DIs have been described for JE virus (Schmaljohn and Blair, 1979) and were detected in yellow fever virus-infected mammalian cell cultures. (C. Rice, personal communication) but in the latter it did not compete the parental virus. So far no DI-like RNA has been observed in viral preparations from yellow fever 17DD virus - infected chick embryo extracts (Santos, Post, Cabral, Souza Lopes and Galler, unpublished). DI RNAs have been successfully used to establish some of the cis-acting sequences required for RNA replication in alphaviruses (Schlesinger and Weiss, 1986) but may be not that useful for flaviviruses.

Regarding the other nonstructural proteins, the NS1 has a structural character given its consistent glycosylation pattern in a number of cell lines (Galler, Post and Rice, unpublished) as shown for yellow fever 17D virus strains and other flaviviruses as well (Winckler et al, 1988) and the number and position of cysteine residues conserved throughout the flaviviruses. It will protect animals against homologous virus challenges if given as a subunit immunogen or as monoclonals against it (Gibson et al, 1988). It is not part of the mature virus and its function in the virus cycle and possibly pathogenesis, if any, is still unknown.

The last 4 nonstructural proteins, ns2a/2b and ns4a/4b have been identified in protein extracts of flavivirus-infected cells (Speight et al, 1988), but establishing their relevance to the virus cycle awaits further research.

Comparative analysis of Flavivirus genomes.

Detailed comparisons of flavivirus genomes has recently become possible due to the accumulation of nucleotide and aminoacid sequences (Rice et al, 1985; Wengler et al, 1985; Deubel et al, 1986; 1988; Zhao et al, 1986; Dalgarno et al, 1986; Trent et al, 1986, Mason et al, 1987; Hahn et al, 1988). A very recent paper (Hahn et al, 1988) gives an overall pattern emerging from the comparisons:

a) at the nucleotide level, strains of the same virus have about 90% homology but up to 96% aminoacid sequence homology.

b) in the same serogroup aminoacid sequence homology drops to 65-70% and necleotide sequences are hardly comparable.

c) members of different serogroups share about 40-50% of aminoacid sequences.

d) short conserved nucleotide sequences have been identified at the 3 end of flavivirus RNA as well as stable secondary structures (Rice et al, 1986; Brinton, 1986; Wengler and Castle, 1986: Hahn et al, 1987b) that could be involved in RNA replication and/or packaging.

e) Several stretches of aminoacids along the genome are perfectly conserved among all flaviviruses analysed so far. However, the nucleotide sequences which code for these strings are divergent as to randomize, but not completely, codon usage.

f) Number of transversions is smaller than transitions suggesting higher rate of misincorporation during replication. In addition transitions in the third codon position are less likely to lead to a change in codon assignment than are transversions and would more likely survive selection pressures.

g) Aminoacid sequences homologies as displayed in a homology plot of any two flaviviruses from different serogroups can vary from nearly 0 to 100% depending on the region of the genome compared. In extreme cases as the nonstructural proteins ns2a/2b and 4a/4b aminoacid sequence homology values are lowest, however, the hydrophobic character of these polypeptides is strikingly conserved.

h) Hidrophobicity/hidrophilicity plots of aminoacid sequences are remarkably similar among flaviviruses despite all the aminoacid sequence divergence.

All in all, it is clear that all the flaviviruses are closely related and have evolved from a common ancestor. There are conserved domains where the aminoacid sequence is important whereas in others the hydrophobic character seems to matter. It is expected that variable and conserved domains can be correlated with specific protein epitopes important for biological properties like cell penetration, interaction with the immune system and so on. These knowledge would be essential for designing second generation dengue vaccines.

Perspectives for the development of dengue vaccines.

The only candidate vaccines for dengue are the dengue 2 PR 159/S1 strain (Bancroft et al, 1984) and a dengue 4 strain (Eckels et al, 1984). In both cases attenuated viruses were obtained by serial passage in mammalian cell culture leading to small plaque temperature sensitive phenotypes. The former yielded low viremia, was not transmitted by mosquitoes and showed some degree of seroconversion specially if volunteers were previously given the yellow fever vaccine. The frequency of side reactions, however, was too high for mass immunization. The latter had low infectivity even for YF-immune volunteers and its genetic instability also led to the discontinuation of its use.

Another group in Thailand, however, has used primary dog kidney (PDK) cells to serially passage and try to attenuate the different dengue serotypes. They have succeeded with dengue 2 virus which is now entering clinical trials and dengue 1 and 4 are being animal tested. Dengue 3 was difficult to propagate in PDK cells but changes in the cell system have overcome this problem. Since different passages have been tested for a number of biological properties sequence analysis of virus pairs could lead to the molecular basis of virulence/attenuation for dengue viruses and possibly for flaviviruses (J.Strauss and R.Shope, personal communication).

The yellow fever system could also serve as a model for virulence/attenuation studies: the virulent strain (Asibi) which gave rise to attenuated (17D) strain and the number of passages in cell culture separating both are well characterized (Theiler and Smith, 1937). Nucleotide sequence analysis of genomic RNA from both viruses led to the identification of a number of aminoacid changes. These would be

responsible for tissue culture adaptation and/or attenuation (Rice et al, 1985; Hahn et al, 1987a). It is anticipated that obtaining an infectious clone for yellow fever virus would allow the identification of the relevant changes for attenuation. This is so because there is an animal system that reflects the infection in man (Monath, 1981), which could be used to test the phenotypes of viruses regenerated from the cloned cDNA bearing mutations made **in vitro.** This infectious clone has not yet been obtained but a full-length cDNA copy covering the whole genome for the YF 17D-204 virus has been constructed (C.Rice, pers.comm.).
It is reasonable to expect that such a methodology, if successful for the YF virus, could be easily applied to attenuating dengue viruses. Full-length infectious clone constructions are being attempted for different dengue virus serotypes in several laboratories. The most serious complication with this approach is the inexistence of an animal system other than man where the different virus phenotypes could be tested and that would reflect the most severe forms of dengue virus infection (DHF/DSS). In addition, the enhancement phenomena argue for a polyvalent vaccine where all 4 serotypes (attenuated or killed forms) are given at once.

It is also of importance to map, and later express, protective epitopes on the surface of flaviviruses. This approach could also help identify enhancing epitopes. There are several methodologies to do that: a) use of specific synthetic oligopeptides whose sequences are derived from cDNA or protein sequencing studies. These peptides are used to immunize animals which are then challenged with a flavivirus. Both envelope and NS1 proteins of Murray Valley (Roehrig, pers.comm.) and dengue 2 (J.Schlesinger, pers.commun.) are being characterized this way. The former has selected sites based on the hydrophilicity peaks whereas the latter is using a set of overlapping oligopeptides. b) monoclonal antibodies are a powerful tool to dissect viral antigens. Several groups have now developed monoclonals against different flaviviruses and this approach was recently reviewed (Heinz, 1986). A neutralization epitope as identified by a monoclonal antibody, has been identified for both YF Asibi and 17D strains by sequencing neutralization scape mutants (Lobigs et al, 1988). The same region was also shown to be a neutralization epitope for MVE virus using synthetic oligopeptides (Roehrig, pers.commun.).

It has long been speculative that the YF 17D virus strain if properly engineered could carry and express dengue virus epitopes. Again the lack of suitable experimental model for dengue is a limitation. In addition, the phenotype of a YF infectious clone when obtained would have to be determined. It remains as a possibility since epitopes of other viruses have now been introduced into a Sindbis virus infectious clone (Rice et al, 1987) with success (J.Strauss, pers.commum.).

It is also possible to map epitopes on flavivirus proteins using bacterial expression vectors. This approach has been used before for other viral proteins (keegan and Collet, 1986; Strebel et al, 1986; Spindler et al, 1984) but is limited to continuous epitopes. Usually a bacterial plasmid vector (or phage as lambda gt11, Young and Davis, 1983) contains a promoter (lambda pL/pR; trpE) and the recombinant protein is obtained as a fusion product between a bacterial protein (betagalactosidase; protein A; triptophan oxidase or the phage MS2 polymerase) with the viral protein domain after appropriate cloning. Screening with antisera allows the identification of recombinants expressing the viral proteins and deletion-expression mapping, which includes DNA sequencing displays the viral epitopes. Using the trpE vectors (Spindler et al, 1984) we have expressed the carboxi terminal half of a dengue 2 virus envelope protein and detected by western blotting common epitope(s) with a dengue 1 virus after probing the filter with an antidengue 1 hyperimmune serum (Muylaert and Galler, unpublished). The identification of such

epitope(s) is of importance in understanding the enhancement phenomena and fusion proteins as well as antibodies against it would be valuable tools to attempt that.

Eukaryotic cell expression of flavivirus proteins has been pursued mainly using vaccina vectors given the potencial of recombinant vaccinia as new live vaccines. The flavivirus proteins expressed include the structurals from dengue 2 S1 strain (J.Strauss, pers.commum.), from yellow fever virus (Rice, Lenches, Galler, Dalrymple and Strauss, in preparation), from dengue 2 Jamaica strain (Deubel, pers.commun.) and from a dengue 4 (C.J.Lai, pers.commum.).

In all cases, synthesis, processing and modification were correct for the structurals but the YF-NS1 protein was not stable or correctly processed since was present in VV-YF recombinant-infected cell extracts in very low levels as compared to the envelope protein (Rice, Lenches, Galler, Dalrymple and Strauss, in preparation). The immunogenicity of all constructs was poor failing to elicit high low levels of neutralizing antibodies, low HI titers and there was no protection upon challenge. Different hosts and inocculation routes were tested with negative results. It was concluded that live recombinant vaccina virus is not an appropriate system to obtain new vaccine candidates.

On the other hand, baculovirus expression of the structural proteins of dengue 4 virus and of the envelope and NS1 separately was satisfactory. Proteins were correctly processed and were, in fact, immunogenic (C. Lai, pers.commun.). Mice were given whole baculovirus-infected insect cell-extracts, recombinant or not. Recovery from insect cell hemolympha would be preferable than whole cell extracts (Maeda et al, 1985). Further experimentation is required before baculovirus-made flavivirus proteins can be established as bona-fide vaccine candidates.

Last but not least, is the possibility of using the purified nonstructural protein 1 (NS1) as a subunit vaccine. This glycoprotein has been shown to protect mice and monkeys against yellow fever and dengue 2 viruses (see Gibson et al, 1988 for a review) after immunization with affinity-purification of NS1 from YF or dengue-infected animal cells. Very low levels of neutralizing antibodies were present in some animals indicating some envelope protein was copurified. A recent observation might help avoiding this problem: there is a secreted form for NS1 in yellow fever infected cell lines (Galler, Post, Santos, Souza Lopes and Rice) in preparation and this has also been shown for dengue and SLE viruses (Winckler et al, 1988). It remains to be shown whether this secreted form will be protective or not. The structural differences between the two NS1 forms lie in the content of sugar residues (Galler and Rice, unpublished) and the role of carbohidrates in the immunogenicity of this protein is not clear. This aspect is under investigation.

Conclusion.

Dengue viruses represent an ever-increasing health problem throughout the world. Safe and effective vaccines have been pursued,but are not yet available. Several approaches to the development of dengue vaccines are being undertaken which include the identification and expression of protective epitopes and the molecular definition of virulence. The construction of infectious clones for different flaviviruses are of importance for the **in vitro** manipulation of pathogenicity and engineering live attenuated dengue vaccines. Subunit vaccines are also considered and adjuvants might turn out to be important in this respect. Most of the current approaches to developing dengue vaccines, and possibly any flavivirus vaccine, were summarized. Altogether, it should provide us with potential candidate

vaccines, be it a live engineered virus or a subunit vaccine or even a recombinat virus vector expressing a flavivirus protein or an epitope thereof. This is in accordance with the recently published World Health Organization Strategic Plan for Dengue (Brandt et al, 1988).

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