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The yellow fever 17D vaccine virus: molecular basis of viral attenuation and its use as an expression vector

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Abstract

The yellow fever (YF) virus is the prototype flavivirus. The use of molecular techniques has unraveled the basic mechanisms of viral genome structure and expression. Recent trends in flavivirus research include the use of infectious clone technology with which it is possible to recover virus from cloned cDNA. Using this technique, mutations can be introduced at any point of the viral genome and their resulting effect on virus phenotype can be assessed. This approach has opened new possibilities to study several biological viral features with special emphasis on the issue of virulence/attenuation of the YF virus. The feasibility of using YF virus 17D vaccine strain, for which infectious cDNA is available, as a vector for the expression of heterologous antigens is reviewed.

Key words
- Flavivirus
- Yellow fever virus
- Attenuation
- Vaccine
- Gene expression

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Introduction

The identification of yellow fever virus

The *Flavivirus* genus, for which yellow fever (YF) virus is the prototype virus, consists of about 70 viruses mostly arthropod-borne, which are transmitted to vertebrates by mosquitoes or ticks. These viruses can be divided into 8 serological subgroups based on cross-neutralization tests. Members of these groups exist in most continents and are responsible for significant human and animal diseases in these areas. The most important are yellow fever virus in the Americas and Africa (1), dengue (DEN) virus with its four serotypes which is spreading throughout the tropics with increasing frequency of the more severe forms of this disease (dengue hemorrhagic fever and dengue shock syndrome) (2) as well as Japanese encephalitis (JE) with its epidemic and endemic profile in Asia (3). Control of flavivirus transmission has been accomplished mainly by vector control measures and vaccination. Approved vaccines are available only for YF using the attenuated live 17D virus, and for tick-borne encephalitis (TBE) and JE, both as inactivated viruses.

In 1925, the Rockefeller Foundation created a committee of YF for West Africa to determine whether YF was similar to the one in the Americas and whether control campaigns such as those carried out in Brazil at the beginning of the century would be effective in Africa as well. It was noted that the African virus was similar to its American counterpart (4) and the success of these in-

vestigators also came from their ability to infect several monkey species with YF virus which was a milestone in laboratory investigation on YF. Not surprisingly, in 1927, two strains of YF virus were isolated which later gave rise to the vaccines used for human immunization: the Asibi strain (5) isolated from a young African named Asibi by passage in Rhesus monkeys (*Macaca mulatta)*, and later the French viscerotropic virus (FVV) isolated from a patient in Senegal (6).

YF vaccine development

In 1935, the Asibi strain was adapted to grow in mouse embryonic tissue (7). After 17 passages the virus, named 17D, was further cultivated until passage 58 in whole chicken embryonic tissue and thereafter, until passage 114, in denervated chicken embryonic tissue only. At this stage, Theiler and Smith (8) showed a marked reduction in viral viscero- and neurotropism when the virus was injected intracerebrally into monkeys. This virus was further subcultured until passages 227 and 229 and these viruses, without human immune serum, were used to immunize 8 human volunteers (9) with satisfactory results, as shown by the absence of adverse reactions and seroconversion for YF within 2 weeks. Larger scale immunization was then carried out in Brazil (10,11).

The present review describes current trends in yellow fever research to understand the molecular basis of yellow fever virus attenuation and the use of YF 17D virus as a vector for heterologous antigens.

Yellow fever virus genome structure and expression

The flaviviruses are spherical viruses 40-60 nm in diameter with an icosahedral capsid which contains a single positive-stranded RNA molecule. Their replication is entirely cytoplasmic and budding in general occurs into the lumen of the rough endoplasmic reticulum (RER) cisternae. With the development of recombinant DNA technology, novel approaches to the understanding of RNA virus genome structure and expression were possible. For flaviviruses the first studies were published in the mid-eighties and included the complete genome sequences of YF (12) and West Nile viruses (WN) (13). Nucleotide and protein sequence data were subsequently obtained by several laboratories and they form the basis for our current knowledge of genome structure and expression.

The YF virus RNA genome consists of 10,862 nucleotides with short 5' (118 nucleotides) and 3' (511 nucleotides) untranslated regions, a 5' cap structure and nonpolyadenylated 3' end. Conserved RNA sequences and secondary structures which may be important for flavivirus replication and/or packaging have been identified (14,15). This single RNA molecule is also the viral message and its translation in the infected cell results in the synthesis of a polyprotein precursor which undergoes post-translational, but possibly also cotranslational, proteolytic processing to generate 10 virus-specific polypeptides. From the 5' terminus the order of the encoded proteins is the following: C-prM/M; E, NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5 (Ref. 12; see Figure 1). The first 3 proteins constitute the structural proteins, i.e., they form the virus together with the packaged RNA molecule and are called capsid (C, 12-14 kDa), membrane (M, and its precursor prM, 18-22 kDa) and envelope (E, 52-54 kDa), all being encoded in the first quarter of the genome. The remainder of the genome encodes the nonstructural proteins (NS) numbered 1 to 5 in the order of synthesis. Three large nonstructural proteins have highly conserved sequences among flaviviruses, NS1 (38-41 kDa), NS3 (68-70 kDa) and NS5 (100-103 kDa). No role has yet been assigned to NS1, but NS3 has been shown to be bifunctional, with a protease activity needed for the processing of the

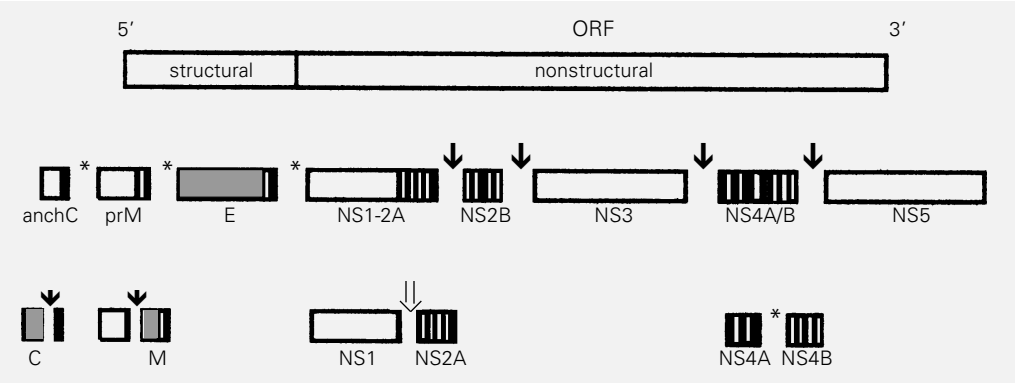

Figure 1 - Schematic presentation of the expression of the flavivirus genome structure. The top represents the whole flavivirus genome with the structural and nonstructural protein coding regions. The boxes below the genome represent precursors and the mature viral proteins generated by proteolytic processing. Shaded boxes represent the structural proteins and open boxes the nonstructural proteins. Black bars represent the stretches of hydrophobic amino acids. Asterisks represent cleavage by cellular signalase; solid arrows, cleavage by the viral NS2B-NS3 complex, including the cleavage of the anchored form of the capsid protein; the open arrow at the NS1/2A cleavage site is a novel still unidentified proteolytic activity.

polyprotein at sites where the cellular proteases will not cleave (16-19) and a nucleotide triphosphatase/helicase activity (20,21) being therefore also associated with viral RNA replication. NS5, the largest and most conserved protein, contains several sequence motifs believed to be common to viral RNA polymerases (14). The 4 small proteins NS2A, NS2B, NS4A and NS4B are poorly conserved in their amino acid sequences but not in their pattern of multiple hydrophobic stretches. NS2A has been shown to be required for proper processing of NS1 (22) whereas NS2B has been shown to associate with the protease activity of NS3 to process itself from NS3 and to produce NS4B (18,23,24). Since viral RNA synthesis takes place in the cytosol in association with RER membranes, it has been postulated that these hydrophobic proteins may be embedded in membranes and, through protein-protein interactions, participate in viral replication complexes together with NS3 and NS5 (12).

Recovery of infectious RNA from cloned cDNA

In order to manipulate RNA genomes, complementary DNA (cDNA) corresponding to the complete genome must be available to allow the introduction of genetic modifications at any particular site of the viral genome. The pioneering study of Racaniello and Baltimore (25) first showed the feasibility to generate virus from cloned cDNA. With the development of *in vitro* transcription systems (26) it became possible to synthesize full length viral RNA *in vitro* with a much higher efficiency when compared to cDNA transcription in the cell. The development of more efficient transfection methods such as cationic liposomes and electroporation helped improve the efficiency of RNA transfection of cultured cells. The basic methodology for what is known today as infectious clone technology now exists. For a number of positive stranded viruses, infectious cDNA has been obtained and can be used to understand the molecular basis of several biological phenomena (Table 1).

Table 1 - Biological properties of flaviviruses.

Virulence/attenuation
Cell penetration
Replication
Host range
Conditional mutants
Design of mutants in genome regions for which no function is known

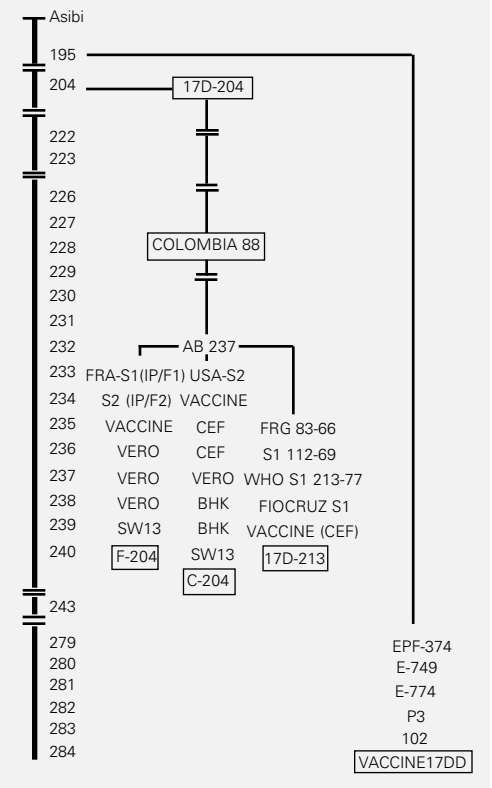

Figure 2 - Passage history of the original YF Asibi strain and derivation of YF 17D vaccine strains. The YF virus Asibi strain was subcultured in embryonic mouse tissue and minced whole chicken embryo with or without nervous tissue. These passages yielded the parent 17D strain at passage 180, 17DD at passage 195, and the 17D-204 at passage 204. 17DD was further subcultured until passage 243 and underwent 43 additional passages in embryonated chicken eggs until the vaccine batch was used for 17DD virus purification (passage 284). 17D-204 was further subcultured to produce the Colombia 88 strain which, upon passage in embryonated chicken eggs, gave rise to different vaccine seed lots currently in use in France (Institut Pasteur, at passage 235) and in the USA (Connaught, at passage 234). Each of these 17D-204 strains was plaque purified in different cell lines, the virus was finally amplified in SW13 cells and used for cDNA cloning and sequence analysis. These 17D-204 substrains are named F-204 and C-204, respectively. The 17D-213 strain was derived from 17D-204 when the primary seed lot (S1 112-69) from the Federal Republic of Germany (FRG 83-66) was used by the World Health Organization (WHO) to produce an avian leukosys virus-free 17D seed (S1 213/77) at passage 237. This 213/77 seed was used to prepare a primary seed at the Oswaldo Cruz Foundation (FIOCRUZ S1) which was passed once more in cultured chicken embryo fibroblasts to produce experimental vaccine batches. The 17D-213 at passage 239 was tested for monkey neurovirulence and was the subject of sequence analysis together with 17DD (at passage 284) and comparison to previously published nucleotide sequences of Asibi (28) and 17D-204 (C-204 (12); F-204 (65)).

Molecular analysis of YF virus attenuation

Structural proteins

Of special interest for vaccine development is the issue of virulence/attenuation but conceivably viral attenuation can result from genetic modification in one or more viral functions. The ideal system to study flavivirus virulence/attenuation is indeed the YF virus for several reasons: a) there is a virulent strain (Asibi) from which an extremely well-characterized vaccine strain was derived (17D, see Figure 2) and which has been successfully used for human vaccination for over 50 years, b) there is an animal system which reflects human infection (27), c) the complete nucleotide sequences of both virulent and attenuated strains have been determined (12,28-30), and d) cDNA clones from which infectious RNA can be synthesized are available (31). The phenotype of YF virus generated from cDNA has been recently determined (32) and shown to be attenuated but slightly neurovirulent for Rhesus monkeys. Therefore, it is possible to design the constructions most informative on virulence determinants mainly by exchanging cDNA segments containing a defined number of mutations, rendering the YF infectious cDNA more Asibi-like and testing the phenotype of the resulting virus in the appropriate animal system. As a complementary approach to the exchange of cDNA sequences between the Asibi and 17D virus genome, the determination of the genomic sequences from other YF vaccine strains should allow us to reduce the number of changes necessary for attenuation and thereby reduce the number of constructions that should be made.

A total of 67 nucleotide differences were originally identified between the Asibi and 17D-204 genomic sequences (28). We have recently determined the genomic sequences from two other YF vaccine 17D substrains,

namely 17DD and 17D-213 (29,30; Figure 2), but not all changes previously identified for the 17D-204 substrain (28) were present and therefore they were not confirmed to be 17D-specific. Therefore, the 17D substrain-specific changes observed are very likely not related to attenuation. As a consequence, the number of changes to be tested in the infectious clone background was reduced by 26%, i.e., to 48 changes. From these 48 nucleotide sequence changes which are scattered along the genome, 26 are silent mutations and 22 led to amino acid substitutions. Although we cannot rule out the importance of changes elsewhere in the genome with regard to viral attenuation, we will restrict this discussion to the alterations identified in the E protein. This protein is the main target for humoral neutralizing response, it is the protein where hemagglutination and neutralization epitopes are located, and it mediates cell receptor recognition and cell penetration, therefore targeting the virus to specific cells. In addition, it accumulated the highest ratio of nonconservative to conservative amino acid changes. Altogether, eleven nucleotide substitutions were observed in the E protein gene leading to 8 amino acid changes.

Alterations at amino acids 52 and 200 are located in domain A of the E protein (domain II in the recently determined 3-D structure proposed for flavivirus E protein; 33) which is conserved among flaviviruses and contains crossreactive epitopes (34). This domain II (A) is highly crosslinked by disulfide bonds and undergoes low pH transition which is related to exposing a strictly conserved and hydrophobic stretch of amino acids believed to be involved in the fusion of the viral envelope to the endosome membrane. This conformational switch leads to the loss of several epitopes recognized by monoclonal antibodies (33). It remains to be seen whether the changes at YF E protein amino acids 52 and 200 affect its ability to fuse with the endosome membrane.

Alterations at amino acids 299, 305, 331 and 380 are located in the B domain (domain III in the 3-D structure; 33). This domain was suggested to be involved in viral attachment to a cellular receptor and consequently to be a major determinant of host range and cell tropism and possibly of virulence/attenuation. The 4 amino acid changes reported for YF (30) are located on the distal face of domain III. This area has a loop which is a tight turn in tick-borne encephalitis virus but contains 4 additional residues in all mosquito-borne strains. Because viruses replicate in their vectors, this loop is likely to be a host range determinant (33). This enlarged loop contains an arginine-glycine-aspartic acid (RGD) sequence in all 3 YF 17D vaccine strains. This sequence motif is known to mediate a number of cell interactions including receptor binding (35). This motif is absent not only in the parental virulent Asibi strain but also in another 22 strains of YF wild type virus (36), again suggesting that the mutation from threonine to arginine creating the RGD motif is likely to be relevant for the attenuated phenotype of the YF 17D strain. It is of interest that Lobigs et al. (37) identified a mutated RGD sequence motif (at amino acid 390) which led to the loss of virulence of Murray Valley encephalitis (MVE) virus for mice. Final proof for the involvement of the envelope protein RGD motif in tissue tropism may come from *in vivo* studies preferably in monkeys since the nonhuman primate model does reflect human disease. It should be kept in mind that there is still no clear definition of virulence determinants for YF virus but the RGD motif might be one.

Alterations at amino acids 170 and 173 in domain C (domain I of the E protein 3-D structure) map very close to the position where a neutralization epitope was identified for TBE virus (34). A mutation at position 171 of TBE virus E protein was shown to affect the threshold of fusion-activating conformational change of this protein and

the 2 changes observed for YF 17D virus may be related to the same phenomenon. It is conceivable that a slower rate of fusion may delay the extent of virus production and thereby lead to a milder infection of the host. It is noteworthy that the recent development of infectious cDNA for JE virus (38) allowed the identification of a mutation (K for E) at amino acid 136 of the E protein which resulted in the loss of neurovirulence for mice (39). This observation implies that domain I is an important area containing a critical determinant of JE virus virulence in contrast to most of the data obtained from the analysis of virulence for several other flaviviruses which suggest that domain III would be the primary site for virulence/attenuation determinants. Nevertheless, this analysis of the E protein provides a framework for understanding several aspects of flavivirus biology and suggests that it should be possible to engineer viruses for the development of new live flavivirus vaccines.

Nonstructural proteins

In addition to the E protein, two nonstructural viral proteins are relevant to the viral attenuation. The NS1 protein is the first nonstructural protein which immediately follows the structural region in the genome. There is evidence for the localization of NS1 on the cell surface (40) and the protein exists in both cell-associated and secreted forms (41). This protein is also known to be a soluble complement-fixing antigen (42). Monoclonal antibodies against NS1 are capable of mediating complement-dependent cytolysis of YF 17D-infected cells suggesting a mechanism for protection *in vivo* (43). Comparison of the NS1 amino acid sequences among flaviviruses displayed conservation especially in the carboxy terminal half and the NS1 evolution rate, as deduced by comparing 2 YF viruses, is one of the lowest along the genome. When the YF 17D virus NS1 amino acid sequence was compared to that of the parental virulent Asibi virus it was noted that a single amino acid change was identified in the amino terminus of NS1 (L→F). It is of interest that mutations which abolished N-linked glycosylation of the YF virus NS1 protein led to a significant reduction of virus yields in cultured cells and mouse neurovirulence (44). The 2 mutated glycosylation sites of YF virus are conserved in most of the flaviviruses for which sequences are available. It would be of interest to mutate the respective sites in the Asibi virus NS1 protein and to determine the effect on virulence.

The next protein in line is NS3. The amino terminal domain of NS3 is a serine protease involved in multiple cleavages of the viral polyprotein (14). It is noteworthy that Chambers et al. (16) described mutations in amino acid residues that constitute the active sites of the serine protease domain of YF virus NS3. These changes were introduced in the full-length YF cDNA and did not permit the replication of YF virus, thus being lethal. Other changes could be devised in such a way as to reduce the protease activity and thereby down-regulate the replicative capability of the virus. It is conceivable that mutations which reduce but do not abolish viral polyprotein processing may have an impact on virus production in the infected cell. The C-terminal two-thirds of NS3 encode a helicase activity which is believed to participate in the replication of the viral RNA and an RNA triphosphatase activity which may be involved in capping of the viral RNA (21). When the NS3 amino acid sequences from both the Asibi and 17D strains of YF virus were compared, 5 nucleotide substitutions in the NS3 gene were identified but only one led to an amino acid substitution. The change at position 485 (D→N) was found in an area which is likely to link the domains of the helicase and RNA triphosphatase.

It was concluded that of the 31 amino acid changes found to be 17D-204-specific, as originally proposed (28), only 22 have been found to be common to the three YF

17D viral strains (17D-204, 17D-213 and 17DD; Ref. 30). Although the other changes, both silent and coding, may also be important in attenuation, fourteen of the 17D-specific changes are arguably nonconservative regarding the character of the substituting amino acid and may be biologically more significant. However, direct testing using recombinant DNA technology to construct progeny viruses bearing specific mutations and their phenotypic characterization in appropriate animal systems will be necessary to fully assess the role of each of these mutations in viral attenuation (31,32).

The identification of a common mechanism for flavivirus attenuation may be feasible, but it is not certain that a given mechanism will hold for every flavivirus. The current trend is to develop infectious clones for all flaviviruses which represent major public health problems and directly study the parameters of viral attenuation for each. Evidently, a number of crippling mutations must be introduced in order to obtain new vaccine candidates. The optimal selection of mutated codons is also important to ensure that the high mutation rate characteristic of RNA viruses (45) will not lead to reversion.

Development of YF 17D virus as an expression vector

As an alternative to developing new vaccines based on the mapping of virulence determinants, the construction of chimeric viruses has become a common approach. The rationale behind it is to have cDNA from a positive-stranded virus with well-known vaccine properties and to use this cDNA as a carrier for heterologous antigens, while ensuring that the virus recovered from cloned cDNA retains the attenuated phenotype. Kohara et al. (46) proposed that an infectious cDNA for the poliovirus Sabin type 1 could be used as stable repository and inoculum for the oral poliovirus vaccine based on the analysis of several phenotypic markers including monkey neurovirulence for both the original vaccine virus and the virus generated from the cloned cDNA.

Development of a YF 17DD infectious clone suitable for YF vaccine production

The phenotypic testing of the virus recovered from the 17D-204 substrain cDNA showed that the virus is suitable for mapping virulence determinants as far as reversion to the wild type phenotype is concerned. However, the slightly higher clinical score observed in neurovirulence tests suggests caution in its use for human vaccination (32). It is also noteworthy that the 204 substrain used for the preparation of the cDNA library and the infectious cDNA (12,31) is closely related to the parental virus used in the derivation of vaccine viruses which caused most cases of encephalitis in vaccinees (47,48). In contrast, no cases of post-vaccine encephalitis have ever been reported for the 17DD substrain, even in the early days of vaccination (49,50). Moreover, 17DD (EPlow) has accumulated fewer nucleotide changes during its passages than the 204 lineage members as deduced from nucleotide sequence comparisons (Santos CND, Post PR and Galler R, unpublished data). Therefore, it would be of interest to try to obtain an infectious cDNA which would be DD-like in its genomic sequence since the DD sequence was determined directly from RNA of virus for which the phenotype has been well established for decades (30). This DD-like cDNA could become a stable repository for the genome of the YF vaccine virus and be the vector of preference if any recombinants prove to have potential as human immunogens in the future. Finally, the propagation of RNA virus vaccine strains as DNA with its correspondingly lower mutation frequency combined with efficient production of vaccine virus in cell culture should improve the quality and lower the cost of the YF live attenuated vaccine.

Use of YF 17D as an expression vector

Expression of whole protein cassettes

Before the need for genetic modification of the present YF infectious cDNA was realized, experiments were conducted to assess the feasibility of using the DNA complementary (cDNA) to the yellow fever vaccine virus genome as a vector for the expression of heterologous antigens. The first chimeric construct attempted with YF cDNA was the precise in-frame replacement of the whole NS1 gene of YF by the equivalent gene of dengue type 2 and the resulting chimera was not viable (Muylaert I, Galler R and Rice C, unpublished data).

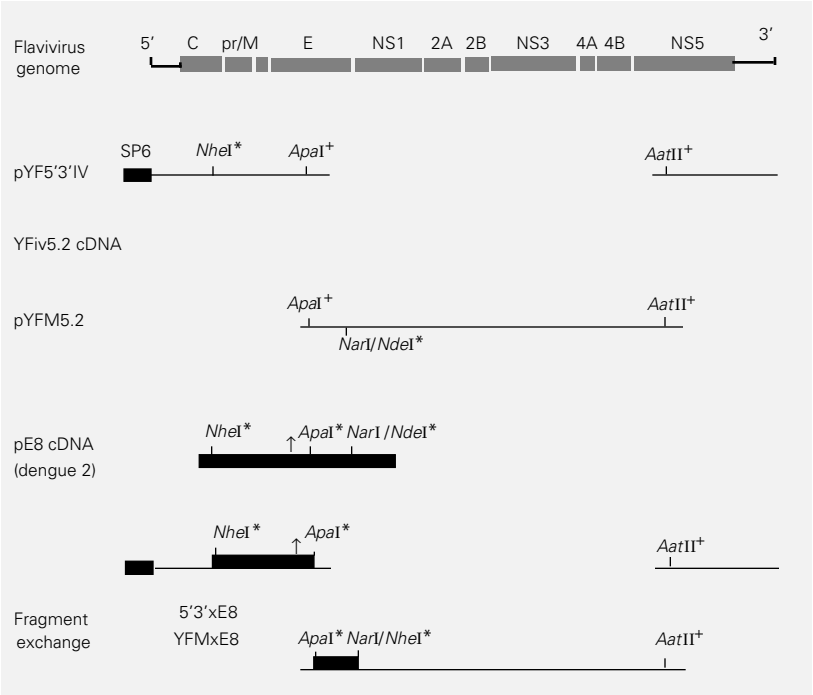

Figure 3 - Strategies for the insertion of dengue virus type 2 sequences into YF cDNA. The top part shows the flavivirus genome (14). The middle part displays the YFiv5.2 cDNA (thin solid lines) which is made up of two separate plasmids (pYF5′3′IV and pYFM5.2) which upon digestion with *Apa*I/*Aat*II and *in vitro* ligation will give rise to infectious RNA transcripts (31) with the resulting virus bearing an attenuated phenotype when compared to the Asibi virus (32). Below the YF cDNA the pE8 cDNA clone is shown encompassing most of the structural region from dengue virus type 2 S1 strain (thick solid lines; 66) in which the arrow represents the N-linked glycosylation site conserved among flaviviruses (14) and present in some strains of YF virus (29). Pre-existing restriction sites (+) and sites created by site-directed mutagenesis (*) are shown. Dengue sequence insertion after site-directed mutagenesis into each of the 2 YF cDNA plasmids is shown in the bottom part as fragment exchange.

Results obtained by other research groups point to the viability of chimeric flaviviruses as intratypic recombinants: the prM/M and E genes from dengue virus serotypes 1, 2 and 3 were used to substitute the dengue virus serotype 4 genome equivalents (51,52). These chimeric viruses induced antibody formation which protected monkeys against a challenge with virulent virus (Lai C-J, personal communication). The construction of intertypic recombinants was also shown to be viable when the structural proteins of TBE virus replaced those of dengue type 4 virus (53). However, there are several disadvantages in the use of these chimeras compared to the use of yellow fever 17D virus, i.e., the inexistence of an animal model which reflects the human infection, such as the genus *Macacca* for YF, the fact that the infectious clone of dengue 4 was derived from a wild type strain, and the lack of well-established production methods or quality tests. Therefore, the use of cDNA-derived yellow fever virus with vaccinal properties would seem more appropriate for the expression of heterologous antigens intended for vaccine development.

We have attempted to construct recombinant plasmids bearing the genome sequences of the yellow fever virus and of two dengue serotype 2 virus strains, the wild type New Guinea C and the vaccinal PR159/S1 strains, both with marked amino acid sequence differences in the structural proteins. In the first recombinant plasmids we developed, based on published results (51,53), YF virus sequences were substituted for dengue virus sequences (see Figure 3). The sequences corresponding to the YF virus structural protein genes prM/M/E were exchanged for the corresponding regions in the dengue 2 virus genome. All attempts to recover the chimeric virus with either PR159/S1 or New Guinea C cDNA failed (Galler R and Ferreira II, unpublished data), suggesting the impracticability of this approach. However, the fusion between the dengue and YF proteins

was made by the creation of a common restriction site in the carboxy terminal region of the C protein (see Figure 3). Recent results indicate that the creation of YF 17D chimeras is indeed possible as a recombinant YF 17D virus bearing the structural prM/E genes of Japanese encephalitis virus was shown to be viable (Mason PW, personal communication). However, the proteins were fused precisely at the cleavage site between C and prM. This seems to be a considerable difference since prM/E subviral particles are produced or not from vaccinia recombinants depending on the extent of sequences preceding the prM amino terminus (Mason PW, personal communication). This approach is seen as a reasonable one to develop YF 17D as an expression vector for heterologous antigens, especially if one considers that the major antigenic protein of YF virus, the E protein, will be absent, and therefore, the criticism based on the pre-existence of antibodies against the vector itself upon repeated immunization may not apply. However, concerns have been raised about safety and ethical issues as far as vaccine development intended for human use is concerned. If nothing else, it is certainly a way of mapping some virulence determinants of flaviviruses (54,55).

Epitope expression by recombinant YF 17D viruses

One possible alternative to continue the analysis of the YF 17D virus as a vector for heterologous antigens is the expression of particular epitopes in certain regions of the genome. For example, neutralizing epitopes to other viral agents could be inserted into regions of the viral envelope where one detects genetic variability by nucleotide sequencing. Such an approach was first tested after replacing 2 amino acids in the envelope protein gene of the YF infectious cDNA with the corresponding amino acid sequence of Murray Valley encephalitis virus (MVE) which was previously characterized by a monoclonal antibody as a neutralizing MVE epitope (Weir R and Rice C, personal communication). The chimera, however, was not viable suggesting that a particular area of the E protein (amino acids 192-193 from the amino terminus) is critical for YF virus viability. The use of viral structural proteins for epitope insertion/expression is not straightforward. For poliovirus, the solution of its three-dimensional structure allowed the mapping of type-specific neutralization epitopes on defined surface regions of the viral particle (56). One of the surface loops of the VP1 protein was used for the insertion of the type 3 epitope which was recognized by primate antisera to poliovirus type 3 showing that the chimera was not only viable but also that the inserted epitope was present in the same conformation as on the surface of the type 3 virus (57). The fact that the 3-D structure for the flavivirus E protein is now available would support the use of this approach for flaviviruses. However, the observation that the same site was used for the insertion of different epitopes of hepatitis A virus but the immunogenicity of the inserted peptides was very poor (58) argues strongly against this approach. Another very simple and elegant approach to the development of chimeric RNA viruses was described by London et al. (59), who inserted a single well-defined neutralizing epitope of Rift Valley fever virus (RVF) into the genome of Sindbis virus by random mutagenesis. Insertion sites, permissive for recovery of viruses with growth properties similar to the parental virus, were found in one of the virion glycoproteins. For these chimeras the epitope was expressed on the virion surface and stimulated a partially protective immune response to RVF infection in mice. The major concern about inserting epitopes into the YF E protein is related to the fact that this protein is the main target for the humoral neutralizing response, the protein where hemagglutination and neutralization epitopes are located,

and that it mediates cell receptor recognition and cell penetration, therefore targeting the virus to specific cells. By inserting a new epitope somewhere in the E protein one or more of these properties could be changed for better or for worse and the vaccine phenotype could be lost or compromised. Nevertheless, it is a system in which the immunogenicity of particular epitopes can be studied.

In this regard, NS3 is of interest and the first candidates for expression are dengue NS3 epitopes. It is an apparently multifunctional nonstructural protein since its has protease activity in its amino terminal third and RNA triphosphatase activity in its carboxy terminal third. It is a hydrophilic protein present in the cytoplasm of infected cells and possibly involved in viral RNA replication together with the putative RNA polymerase (NS5). Several cytotoxic T cell (CTL) epitopes have been identified in the NS3 protein of flaviviruses, such as dengue, West Nile and Murray Valley encephalitis (60-64).

Even though reactive CTLs are identified in individuals recovered from dengue virus infection and CTL epitopes have been recently identified by Livingston et al. (64), their role in protective immunity against viral infection or immunopathology in infected individuals is not clear. However, if dengue virus-specific epitopes could be expressed using recombinant YF virus, *in vivo* evaluation of epitope functional expression could be performed in terms of the presence of antibodies and CTLs reactive with the recombinant virus by using human biological samples obtained from individuals during acute disease or convalescence. Alternatively, the protective role of such CTL epitopes could be verified by the inability of prototype dengue virus to cause viremia and fever in recipient monkeys previously infected with the recombinant virus as compared to controls inoculated with YF virus only or not previously inoculated. The intention is to establish the viability of such an approach by studying the immune response to dengue virus, a system that poses several basic questions regarding infectious diseases.

Cytotoxic T cells, mostly $CD8^+$, which require the class I antigen presentation pathway are primarily generated by intracellular microbial infections, and have been most thoroughly investigated in viral infections. Recombinant viruses expressing the desired foreign epitopes are therefore a logical approach for generating the cytotoxic T cells of the desired specificity. We now propose to explore the feasibility of using the YF 17D virus, not only as a very effective proven yellow fever vaccine, but also as a vector for protective antigens (epitopes). This approch, we hope, will result in the development of a vaccine which is effective against yellow fever and other diseases which may occur in the same geographical areas.

Conclusion

The application of molecular techniques to the study of flavivirus genome structure and expression provides new approaches to the understanding of viral biology mainly by the use of the infectious cDNA technology. This technology has already identified some mechanisms which may lead to a virus altered at specific points of the viral cycle. Attenuation can be effected by introducing multiple changes and making the mutant less prone to reversion given the high mutation rate of RNA viruses. These mutants could be used for experimental infections if the appropriate animal models are available and may result in viruses with potential as new live vaccines. The yellow fever virus, despite a long period of being ignored by researchers, probably due to the effectiveness of the vaccine, has made a comeback both in nature as human populations grow and reach endemic areas and in the laboratory, being a suitable model for understanding the biology of flaviviruses in general and providing new alternatives for vaccine development.

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