

## DSM Science & Technology Awards 2004

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## Summary of the candidate's work

Flaviviruses are among the most important emerging viruses known to man. The flavivirus genus consists of nearly 80 viruses, which are phylogenetically grouped into three clusters: viruses that are transmitted (i) by mosquitoes or (ii) by ticks (arthropod-borne) and (iii) the so called viruses with not known vector (NKV) (1).

Flaviviruses cause a variety of diseases including encephalitis and (hemorrhagic) fevers. One of the most important flaviviruses causing human disease is dengue virus (DENV). It is estimated that there are worldwide annually as many as 50 to 100 million cases of dengue fever and several hundred thousand cases of dengue hemorrhagic fever (DHF), the latter with an overall case fatality rate of ~5% (6,11). Despite the availability of a very efficacious vaccine, yellow fever (YF) is still a major public health problem. The World Health Organization estimates that there are annually 200,000 cases of YF, including 30,000 deaths, of which over 90% occur in Africa. Japanese encephalitis (JE), a mosquito-borne arboviral infection, is the leading cause of viral encephalitis in Asia (7). Approximately 50,000 sporadic and epidemic cases of JE are reported annually and result in high mortality (30%), while ~30% of the survivors develop long-lasting neurological sequelae (8,10). Other important flaviviruses that cause encephalitis are also responsible for high mortality rates or neurological sequelae, such as tick-borne encephalitis virus (TBEV), West Nile virus (WNV), St. Louis encephalitis virus (SLEV). More than 30% of patients that are hospitalized with flavivirus encephalitis, do not survive the infection. About 50% of the patients that recover from the infection, have permanent physical or psychological disorders.

Currently, there is no antiviral therapy available for the treatment of infections with flaviviruses and such therapy is not expected soon. The search for (novel) strategies for the treatment of flavivirus infections has been hampered, in part, by the absence of a convenient small animal model. Monkeys infected with flaviviruses such as YFV, DENV or JEV may develop infection, but because of the cost involved and the restricted availability of monkeys, the number of studies that can be carried out by using these animals is limited. Some flaviviruses (e.g. DENV, YFV) cause morbidity and mortality in mice, but only when inoculated intracerebrally and when virus strains are used that have been adapted by serial passage in the brain of suckling mice. Other flaviviruses may cause infection in mice following peripheral inoculation, although these viruses require special research facilities,

e.g. biosafety level 3 (BSL3) conditions for manipulation of JEV, and even BSL4 containment facilities for tick-borne encephalitis viruses, which is an obstacle for intensive research with animals.

Ideally, a virus used in a model for the evaluation of novel antiviral strategies should (i) cause morbidity and mortality in small (adult) laboratory animals following systemic inoculation, (ii) not be pathogenic to man, and (iii) mimick human disease.

Both Modoc virus and Montana *Myotis* leukoencephalitis virus fulfill these criteria. MODV is a murine flavivirus that was originally isolated from white-footed deer mice (*Peromyscus maniculatus*) collected in Modoc county, California, in 1958. The bat flavivirus MMLV was first isolated in 1958 from a mouse bitten in a laboratory condition by a naturally infected little brown bat (*Myotis lucifugus*) captured in Western Montana. Both flaviviruses have been assigned to the cluster of the not known vector (NKV) flaviviruses.

It was our aim to develop novel models for the study of antiviral therapy against flavivirus infections in rodents. Therefore, we sequenced and characterized the complete genome of MMLV and studied the characteristics of this virus *in vitro* and *in vivo*. Furthermore, we wanted to render flaviviruses that cause disease in man (such as YFV and DENV), but that are not infectious to adult mice upon systemic inoculation, infectious to mice. We also hypothesized that detailed comparison of the viruses belonging to the arthropod-borne and the NKV cluster should allow to define the determinants that are responsible for the fact that a given flavivirus is either able or not to replicate in cells of a vector (mosquitoes or ticks).

## **1. The genome of MMLV, a NKV flavivirus**

The MMLV genome shares the same overall characteristics with flaviviruses of human importance. First, MMLV has a similar genomic organization, i.e. the complete genome is 10,690 nucleotides long and contains one long open reading frame encoding a putative polyprotein of 3374 amino acids, that is flanked by a 5' and 3' UTR of, respectively, 108 and 457 nucleotides long. The coding region contains homologous protease cleavage sites, internal signal sequences and transmembrane sequences. Furthermore, the virus contains the same conserved motifs, in genes that are believed to be interesting antiviral targets [NTPase/helicase, serine protease and RNA-dependent RNA polymerase],

as in flaviviruses that cause disease in man. As a consequence, MMLV may serve in antiviral drug studies as a good surrogate for flaviviruses that are of medical importance.

So far, very little is known about the 3' UTR of the NKV flaviviruses. We therefore performed a comparative analysis of the RNA folding of the 3' UTR of the NKV flaviviruses (in addition to MMLV, we used Modoc, Rio Bravo and Apoi viruses). Structural elements in the 3' UTR that are preserved among other flaviviruses have been revealed, as well as elements that distinguish the NKV from the mosquito- and tick-borne flaviviruses. Most importantly, the pentanucleotide sequence 5'-CACAG-3', which is conserved in all mosquito- and tick-borne flaviviruses, is replaced by the sequence 5'-C(C/U)(C/U)AG-3' in the loop of the 3' long stable hairpin structure of all four NKV flaviviruses studied. Knowledge of this pentanucleotide of flaviviruses thus allows to classify a flavivirus as either a vector-borne or NKV flavivirus (3). The phenotypical implications of this altered pentanucleotide motif were further investigated (see Chapter 4).

## **2. The MMLV mouse model**

We next employed MMLV to elaborate a convenient flavivirus animal model. So far, no infections in humans have been reported for this virus, which points to the interesting safety features of this virus for manipulation in a research laboratory.

MMLV replicates well in Vero cells, which makes the virus convenient to use in cell culture systems and therefore for antiviral evaluation. Cells infected with MMLV show dilatation of the endoplasmic reticulum, a characteristic of flavivirus infection. The virus appears to be equally sensitive as flaviviruses of human importance (i.e. YFV and DENV) to a selection of (experimental) antiviral agents.

Experimental inoculation (intraperitoneal, intranasal or direct intracerebral) of immunodeficient mice with MMLV resulted in encephalitis ultimately leading to death. Viral RNA and/or antigens were detected in the brain and serum of MMLV-infected SCID (Severe Combined Immuno Deficiency) mice, but not in any other organ examined. In the brain, MMLV was detected in the olfactory lobes, the cerebral cortex, the limbic structures, the midbrain, *cerebellum* and *medulla oblongata*. Infection was confined to neurons. To validate the MMLV-model for use in antiviral drug studies, we treated SCID mice with the interferon  $\alpha/\beta$  inducer poly(I).poly(C) before inoculation with MMLV. The mice were

protected against MMLV-induced morbidity and mortality, and this protection correlated with a reduction in infectious virus titer and viral RNA load. This validates the MMLV-mouse model for the study of chemoprophylactic or chemotherapeutic strategies against flavivirus infections causing encephalitis (2).

### **3. Construction and characterization of a chimeric MODV/YFV flavivirus**

As mentioned above, YFV is not infectious to small animals, which hampers the *in vivo* evaluation of potential novel treatment strategies. The aim of our research project was to construct a YFV that is infectious to mice, by exchanging the envelope proteins of the vaccine strain of yellow fever (YFV 17D) with those of a murine flavivirus, i.e. MODV (which is infectious to mice, but not to man) (4).

So far, full-length cDNAs of chimeric flaviviruses have been constructed by restriction-enzyme cleavage of the gene(s) to be exchanged or by fusion-PCR of two amplified PCR fragments. Compared to the restriction method, which requires the incorporation of mutations to create unique restriction sites, the fusion-PCR strategy has the advantage that the gene fusion can be made at any position within the nucleotide sequence. We developed a new, faster and straightforward variant on the classical fusion-PCR method for the construction of chimeric (flavi)viruses. Instead of three amplification reactions with four primers as used in a “classical fusion reaction”, a fusion between two different genes was accomplished by only two amplification reactions (and the use of three primers), thereby reducing the risk of accumulation of mutations.

The resulting MODV/YFV chimeric virus was characterized *in vitro* and *in vivo*. MODV/YFV replicated (in Vero cells) about as efficiently as the parental viruses. In SCID mice that had been infected intraperitoneally with the chimeric virus, viremia developed at day 3 post infection, and the viral load increased steadily. The MODV/YFV virus, like MODV from which it had acquired the prM and E envelope proteins, proved neuroinvasive in suckling and adult SCID mice, in contrast to YFV(17D). All animals developed neurological symptoms (including paralysis) and showed 100% mortality. The distribution of MODV/YFV RNA, as detected by *in situ* hybridisation, was similar to that observed in MODV-infected mice. These observations provide for the first time compelling evidence that the determinants of neuroinvasiveness of flaviviruses are entirely located in the

envelope proteins prM and E. Furthermore, we demonstrate in this study that by adapting YFV by chimerization, a flavivirus that is responsible for disease in man, can be made infectious to mice (following infection by the intraperitoneal route). This chimeric virus contains the entire original replication machinery of YFV and can thus be used to study antiviral strategies against YFV in a mouse model (5).

This information may be instrumental to further understand the molecular basis of flavivirus neuroinvasiveness and, may have implications for engineering live attenuated chimeric vaccines

#### **4. Identification of the factor that determines transmission of a flavivirus by a vector**

So far no information is available on which factor(s) determines(s) whether a flavivirus is able to replicate in a vector (mosquito or tick) or not. The MODV/YFV virus, described above, is the first chimeric virus between an arthropod-borne and a NKV flavivirus. Since MODV is not infectious to mosquito or tick cells (our unpublished observations), we hypothesized that also the chimeric MODV/YFV, which contains the envelope proteins of MODV, would not be infectious to mosquito cells. However, MODV/YFV replicated as efficiently as YFV in mosquito cells. This implicates that determinants other than prM+E in the viral genome are responsible for the fact that a flavivirus is vector-borne or a non vector-borne.

In Chapter 1 we identified an altered pentanucleotide motif in the genome of NKV flaviviruses when compared to vector-borne flaviviruses. The very 3' terminus of the 3' UTR of the flavivirus genome folds in a manner typical for all flaviviruses, forming a 3' LSH structure and a small stem-loop. It is suggested that the 3' LSH plays a crucial role in the replication of all flaviviruses. In particular, the presence of the highly conserved pentanucleotide 5'-CACAG-3' in the top loop has been suggested to play an important role in virus replication (9). Analysis of the 3' UTR sequence of NKV flaviviruses revealed that the pentanucleotide sequence (5'-CACAG-3') which is conserved in all mosquito- and tick-borne flaviviruses, consists of the sequence 5'-C(C/U)(C/U)AG-3' in the genome of all four NKV flaviviruses. Because prM+E has no effect on vector specificity, we investigated the possible impact of this motif on the vector specificity of flaviviruses. We therefore carried out site-directed mutagenesis on the pentanucleotide sequence of the chimeric MODV/YFV

virus, and evaluated whether the resulting mutant [named MODV/YFV(CUCAG)] is able or not to replicate in mosquito (C6/36) cells. The replication kinetics of MODV/YFV(CUCAG) were compared with those of MODV, YFV 17D and the original chimeric MODV/YFV. The MODV/YFV(CUCAG) replicated as efficiently in Vero cells as MODV, YFV 17D and MODV/YFV(CACAG), but failed to replicate in mosquito cells in contrast to MODV/YFV(CACAG). These data provide strong evidence that the pentanucleotide motif in the 3' LSH determines whether a flavivirus is able to replicate in mosquito cells or not. The practical consequence of this finding is that it may allow to destroy the ability of a live attenuated flavivirus to be transferred by a vector from host (man) to host. This intriguing finding has been patented.

To further confirm this hypothesis, we are currently constructing a YFV 17D virus that contains the CUCAG sequence. Also will it be important to assess whether mutating the pentanucleotide motif CUCAG of a NKV flavivirus to the CACAG sequence of vector-borne flaviviruses results in NKV viruses that are infectious to vector (mosquito or tick) cells. To this end, we need to start from full-length infectious clones of NKV flaviviruses. Such clones do not exist and we therefore decided to generate such clone.

## **5. Construction and development of a full-length infectious Modoc virus clone**

Infectious clone technology offers a modern and direct approach to analyse and modify viral genomes at the molecular level. Infectious flavivirus clones have been developed for several arthropod-borne flaviviruses (such as YFV, JEV, DENV, TBEV). However, no infectious full-length clone is available for any of the NKV flaviviruses.

A problem in the construction of infectious clones of flaviviruses is the synthesis of full-length cDNA. To address this problem we have devised a rapid and simple method to generate a full-length long RT-PCR product using viral RNA isolated from either (i) the supernatant of MODV-infected cells or (ii) an unpurified MODV-infected mouse brain suspension. These developments represent a significant advance in recombinant technology and the first method should be applicable to positive-stranded RNA viruses which do not replicate to high titers in mouse brain.

The availability of a full-length infectious NKV flavivirus (MODV) clone creates new opportunities for flavivirus research. It will allow to investigate whether changing the

CUCAG motif of MODV into the arthropod-borne motif, results in a virus that is able to replicate in mosquito or tick cells (and mosquitoes or ticks) (see also Chapter 4). Furthermore, such clone can be used to unravel molecular mechanisms that play a role in the replication of the viral genome and assembly of viral particles. Also, by inserting the green fluorescent protein (GFP) gene in the genome of MODV, the pathogenesis and neurotropism of flaviviruses can be studied in detail. Also chimeric flaviviruses can be constructed, based on the MODV backbone, in which certain genes have been exchanged by the homologous genes of other Flaviviridae.

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