167167

Flavivirus NS1 Subunit Vaccine

The present invention relates to NS1 proteins or parts thereof of Flaviviruses, in particular of Dengue viruses useful for vaccination against said Flavivirus and against one or more other Flaviviruses. The invention further concerns the NS1 protein or parts thereof of one Dengue virus serotype, in particular serotype 2, useful for vaccination against Dengue viruses from all serotypes. The invention further concerns DNA comprising an expression cassette coding for a Flavivirus NS1 or parts thereof, vectors comprising said DNA and vaccines containing or expressing a Flavivirus NS1.

Background of the invention

The etiological agent of the dengue fever is the Dengue virus, belonging to the Flavivirus genus of the family Flaviviridae (Burke and Monath, 2001). A particularly important subgroup of Flaviviruses is the group of so called mosquito-borne Flaviviruses, i.e. Flaviviruses that are transmitted by mosquitos. This group comprises in addition to the above mentioned Denguevirus other important viruses such as the West nile virus, the Japanese encephalitis virus and the Yellow fever virus (Fields Virology, ed. by Fields B.N., Lippincott-Raven Publishers, 3rd edition 1996, ISBN: 0-7817-0253-4, pages 931-1034). Typical diseases transmitted by these viruses are West nile fever and West nile encephalitis induced by the West nile virus, encephalitis induced by the Japanese encephalitis virus, Yellow fever induced by the Yellow fever virus and Dengue fever, dengue hemorrhagic fever (DHF; see below) and Dengue shock syndrome (DSS) induced by the Dengue virus.

Flaviviruses are enveloped, single-stranded, positive-sense RNA viruses formed by three structural proteins: the capsid protein (C) that

G00002

forms a nucleocapsid in association with the viral genome, which is surrounded by a lipid bilayer in which are anchored the M (membrane) and E (envelope) proteins. The genome is approximately 11kb long and contains a single open reading frame encoding a polyprotein precursor of about 3400 amino acid residues. Individual viral proteins are generated from this precursor by the action of cellular and viral proteases. The three structural proteins (C, M and E) are derived from the N-terminal part of the polyprotein and are followed by seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Lindenbach and Rice, 2001).

Glycoprotein NS1, present in all Flaviviruses, appears to be essential for virus viability. Dengue virus NS1 is secreted from mammalian infected cells in a soluble hexameric form (Flamand et al., 1999). This noncovalently bound hexameric complex is formed by 3 dimeric subunits and has a molecular mass of 310 kDa. Dimerization is a prerequisite for NS1 protein export to the plasma membrane, where it remains as the unique viral resident protein of the infected cell surface.

In mammalian cells, but not in insect cell lines that support dengue infection, part of the transported NS1 is released into the extracellular milieu. Extracellular NS1 is secreted either as a soluble protein, which exist in a higher hexameric oligomeric form, or in association with microparticles but not with virions. In addition, NS1 has been found circulating in sera from dengue virus infected patients, suggesting that secretion of NS1 may be an important event in Flavivirus infection in the human host. During the course of a Flavivirus infection, the NS1 protein evokes a strong antibody response, which helps to clear the infecting virus from the host, presumably through a complement-mediated pathway (Schlesinger, J.J. et al., 1987) and antibody-dependant cell cytotoxicity (ADCC) (Schlesinger, J.J. et al., 1993).

The Dengue virus, with its four serotypes Dengue virus serotype 1 (Den-1) to Dengue virus serotype 4 (Den-4), is the most important member of the Flavivirus genus with respect to infections of humans and produces diseases that range from flu-like symptoms to severe or fatal illness, dengue haemorrhagic fever with shock syndrome. Dengue outbreaks continue to be a major public health problem in densely populated areas of the tropical and subtropical regions, where mosquito vectors are abundant.

The concern over the spread of dengue infection and other diseases induced by mosquito-borne Flaviviruses in many parts of the world has resulted in more efforts being made towards the development of dengue vaccines, which could prevent both dengue fever (DF), and dengue hemorrhagic fever (DHF) and in vaccines useful to protect the vaccinated individual against infections induced by some or all mosquito-borne Flaviviruses.

While most cases of DF are manifested after the first infection by any of the four serotypes, a large percentage of DHF cases occur in subjects who are infected for the second time by a serotype which is different from the first infecting serotype of dengue virus. These observations give rise to the hypothesis that sequential infection of an individual with antibody against one dengue serotype by a different virus serotype at an appropriate interval may result in DHF in a certain number of cases. Antibody-dependant enhancement (ADE) has been demonstrated *in vitro* for dengue viruses, as well as other enveloped viruses, and is considered to be an important mechanism in the pathogenesis of DHF.

It has also been observed that DHF usually emerges in geographic areas where multiple (three or four) virus serotypes co-circulate. In

regions with endemic DHF such as Southeast Asian countries, the age-specific attack rate is higher in children, and the number of DHF cases decreases in higher age groups. This roughly corresponds with the increasing seroprevalence to dengue, indicating that natural infection may evoke protective immunity. This phenomenon is not unlike that observed with other viral infections such as hepatitis A virus. Anecdotal clinical observations have shown that patients may experience DHF twice (Nimmannitya *et al.*, 1990) but this is rare, and it is difficult to identify accurately the serotypes causing the second and subsequent infections. So far, there has been no reports of a forth infection in the same individual, despite the fact that all four dengue virus serotypes circulate in the same area. This suggests that, in nature, infection by two or three dengue virus serotypes in the same individual may result in cross-reactive antibodies or even a cross-reactive cytotoxic lymphocyte response. This may modulate or protect against infection by the remaining dengue virus serotypes in nature.

At present there are no approved dengue vaccines. Today, prevention of dengue virus infection is dependent upon control of the principal mosquito vector, Aedes aegypti. Insecticide resistance, lack of technical and financial support that would enable local health departments to maintain effective mosquito control programs, and continuing geographic spread of both the vector mosquitoes and dengue viruses make it practically impossible to prevent dengue infections by current mosquito control programs. Therefore, development of safe and effective vaccines against all four serotypes of dengue virus has been designated by the WHO as a priority for the most cost-effective means to prevent dengue virus infection. The WHO has recommended that the ideal vaccine against dengue and DHF should be the kind that can prevent infection caused by all serotypes so that sequential infection cannot happen.

To this end WO 98/13500 proposes to use a recombinant Modified Vaccinia Virus Ankara (MVA) expressing antigens from all Dengue virus serotypes or to use four recombinant MVA wherein each of the recombinant MVA expresses at least one antigen of one Dengue virus serotype. Both strategies provide very promising strategies to vaccinate against all Dengue virus serotypes. However, it is desirable to provide a single subunit vaccine that upon administration results in an immune response against more than one Flavivirus or against more than one serotype of Dengue virus, preferably against all Dengue virus serotypes. Moreover, WO 98/13500 discloses a recombinant MVA encoding Dengue virus NS1. WO 98/13500 does not disclose that an antigen derived from one Dengue virus serotype elicits an immune response not only against the Dengue virus serotype from which the antigen is derived but also against antigens derived from other Dengue virus serotypes.

WO 99/15692 discloses a recombinant MVA containing and capable of expressing one or more DNA sequences encoding Dengue virus antigens not able to effect immune enhancement or antibody dependant enhancement. WO 99/15692 does not disclose that an antigen derived from one Dengue virus serotype elicits an immune response not only against the Dengue virus serotype from which the antigen is derived but also against antigens derived from other Dengue virus serotypes.

Object of the invention

Thus, it is an object of the invention to provide a vaccine derived from a Flavivirus or a Flavivirus serotype that is stable, can easily be produced and that leads to an immune response that protects the vaccinated individual not only against the Flavivirus or the Flavivirus serotype from which the vaccine is derived but also against other Flaviviruses or

Flavivirus serotypes. It is a particular object of the present invention to provide a vaccine derived from a mosquito-borne Flavivirus that protects the vaccinated individual not only against the mosquito-borne Flavivirus or Flavivirus serotype from which the vaccine is derived but also against other mosquito-borne Flaviviruses or Flavivirus serotypes. It is a further object to provide a vaccine that is derived from one Dengue virus serotype and that protects an individual against an infection with at least two, preferably all Dengue virus serotypes.

Detailed description of the invention

These objects have been solved using the NS1 protein or parts thereof of a Flavivirus and DNA sequences comprising an expression cassette coding for a Flavivirus NS1 protein or a part thereof, respectively. In particular, the object to provide a vaccine that is derived from one mosquito-borne Flavivirus and that protects an individual against an infection with the mosquito-borne Flavivirus from which the vaccine is derived but also against an infection with at least one other mosquito-borne Flaviviruses has been solved by using the NS1 protein or a part thereof of a mosquito-borne Flavivirus, in particular the Dengue virus, preferably Dengue virus serotype 2 and corresponding DNA sequences, respectively. More specifically the object to provide a vaccine that is derived from one Dengue virus serotype and that protects an individual at least against an infection with at least two, preferably at least three, more preferably all Dengue virus serotypes and preferably also against the infection with other Flaviviruses, in particular mosquito-borne Flaviviruses such as the Japanese encephalitis virus, the Yellow fever virus and West Nile virus has been solved by using the NS1 protein or a part thereof of a Dengue virus, in particular of Dengue virus serotype 2 and corresponding DNA sequences, respectively.

As it is shown in more detail in the experiment section the NS1 protein derived from a Dengue virus of one serotype expressed *de novo* after vaccination can evoke an antibody response that will cross react with NS1 proteins of Dengue virus serotype 1, 2, 3 and 4 plus NS1 from other members of the Flavivirus genus such as Japanese encephalitis virus, Yellow fever virus and West Nile virus. Thus, NS1 protein from one Dengue virus serotype origin is a universal DHF subunit vaccine for simultaneous protection against at least two, more preferably three, even more preferably all four serotypes of dengue virus and further against one or more other viruses of the genus Flavivirus. Since in this subunit vaccine strategy no E protein is involved, there should be no risk of Antibody Dependant Enhancement (ADE) upon subsequent exposure to any of the serotypes of dengue and therefore no vaccine related DHF should be induced during natural outbreaks of dengue infection.

The NS1 protein may be expressed from a nucleic acid, preferably a DNA comprising an expression cassette coding for at least a Flavivirus NS1 protein or a part thereof. The term "at least" in this context is to be interpreted in that the expression cassette may further encode additional proteins/peptides, either as separate proteins/peptides or fused to the NS1 protein or part thereof as defined in more detail below. In the context of the present invention the term „DNA" refers to any type of DNA, such a single stranded DNA, double stranded DNA, linear or circular DNA or DNA in the form of a plasmid or a viral genome. Since Flaviviruses are RNA viruses the DNA coding for the Flavivirus NS1 protein is a non-naturally occurring DNA, such as a cDNA or a synthetic DNA.

The term „expression cassette coding for a Flavivirus NS1 protein or part thereof" is to be interpreted in that the coding sequence of a Flavivirus NS1 protein or a part thereof is preceded by elements controlling the transcription, in particular the initiation of transcription.

Examples for such transcriptional regulatory elements are prokaryotic promoters and eukaryotic promoter/enhancers. Preferred eukaryotic promoter/enhancers are the human Cytomegalovirus immediate early promoter/enhancer and poxvirus promoters such as the 7.5 promoter and the poxvirus minimal promoter as disclosed in the example section. The sequence of the poxvirus minimal promoter is shown in Figure 2 as well as in SEQ:ID No. 9. The expression cassette may further contain elements controlling the termination of transcription such as prokaryotic termination elements or eukaryotic poly A signal sequences, if necessary.

The expression cassette may express only the NS1 protein or part thereof of a Flavivirus or may express the NS1 protein or part thereof together with one or more further Flavivirus proteins/peptides, wherein the NS1 protein or part thereof and the further proteins/peptides are produced as separate proteins/peptides or as fusion proteins/peptides. If not defined otherwise in this description the term „peptide" in the context of the present invention refers to a contiguous amino acid sequence stretch of at least 10 amino acids, more preferably of at least 20 amino acids, most preferably of at least 25 amino acids.

The further Flavivirus protein is not the entire E-protein since this protein seems to be involved in the development of DHF. Thus, if the further Flavivirus peptide is derived from the E-protein it should comprise less than 40 amino acids, preferably less than 35 amino acids. If amino acid sequence stretches derived from the E-protein are expressed together with the NS1 protein or part thereof it should have been verified that this amino acid stretch does not comprise an epitope that is involved in the generation of ADE and DHF.

If the expression cassette expresses in addition to the NS1 protein or part thereof a further Flavivirus protein/peptide as separate

proteins/peptides the expression cassette may comprise an Internal ribosome entry site (IRES) between the sequence encoding the NS1 protein or part thereof and the sequence encoding the further Flavivirus protein. IRES elements are known to the person skilled in the art. Examples for IRES elements are the picornaviral IRES elements or the 5´non-coding region of the hepatitis C virus.

Alternatively the nucleotide sequence encoding the NS1 protein or part thereof may be fused to a DNA sequence encoding further Flavivirus proteins/peptides in such a way that a fusion protein between the NS1 protein or part thereof and the further Flavivirus protein/peptide is produced. If the NS1 protein or part thereof and the further Flavivirus protein/peptide are to be produced as fusion proteins/peptides the respective coding sequences are fused in frame.

In a preferred embodiment the DNA sequence encoding the NS1 protein or part thereof is preceded by the sequence encoding the glycosylation signal sequence of the E-protein. According to this embodiment a fusion protein is produced that comprises the E-protein gylcosylation signal sequence fused to the NS1 protein or part thereof. As pointed out above the E-protein derived amino acid stretch should be as short as possible and it should be excluded that this amino acid stretch contains an epitope involved in the generation of ADE and DHF. The gylcosylation signal sequence of the E-protein fulfils these requirements.

In an alternative preferred embodiment the expression cassette used according to the present invention contains as only Flavivirus sequence the sequence encoding the NS1 protein or part thereof. Thus, in this preferred embodiment the expression cassette according to the present invention does not express any other peptides/proteins from

other parts of the Flavivirus genome, in particular not the NS2A or the E protein.

In a further alternative embodiment the DNA according to the present invention expresses the NS1 protein or part thereof as a fusion protein with proteins/peptides that are not derived from a flavivirus. Such proteins/peptides comprise non-flaviviral signal sequences or sequences that are useful for the detection or purification of the expressed fusion protein, such as tags.

To understand the general structure of the Flavivirus sequence in the expression cassette used according to a preferred embodiment of the present invention it is helpful to summarize briefly the genome structure of Flaviviruses: During a natural Flavivirus infection the virus produces a single polyprotein which is then cleaved first by host cell proteases and then virus encoded proteases into the following proteins: C, PrM and M, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (protein order along the polyprotein precursor). Therefore, a DNA sequence, in particular a cDNA sequence coding for the NS1 protein or part thereof must require the addition of a "ATG" start codon. In a preferred embodiment the start ATG is then followed by a sequence encoding a glycosylation signal so that the newly synthesized NS1 protein becomes glycosylated in the endoplasmic reticulum. Such signal sequences are known to the person skilled in the art. Finally, the protein-coding cassette needs a stop codon, which might be a TAG added to the 3' terminal end of the protein coding cDNA sequence. In the example used in this invention the "ATG+signal sequence" element was derived from the sequence encoding the hydrophobic C-terminal end of the E protein (the last 28 amino acids, which for the Dengue virus New Guinea strain („NGC strain", GeneBank accession number AF038403) starts with the amino acid M (ATG). A

typical expression cassette according to the present invention is shown in Figure 2 and as SEQ:ID No 9 and SEQ:ID No. 10.

Thus, in summary this embodiment concerns the use of a DNA comprising an expression cassette comprising the sequences coding for a Flavivirus NS1 protein or part thereof, wherein the coding sequence is preceded by a start codon ("ATG") and a sequence encoding a signal sequence for glycosylation, preferably derived from the E-protein as defined above and wherein the coding sequence is terminated by a stop codon of translation (Figures 1A, 1C and 2, SEQ:ID 5-10).

The DNA sequence that may be used according to the present invention encodes a Flavivirus NS1 protein or part thereof. The term "Flavivirus" refers to any Flavivirus. More preferably the term "Flavivirus" refers to mosquito-borne Flaviviruses such as the West nile virus, the Japanese encephalitis virus, the Yellow fever virus and the Dengue virus. The NS1 protein or part thereof derived from one mosquito-borne virus encoded by a DNA according to the present invention should protect the vaccinated individual not only against an infection with the virus or virus serotype from which the vaccine is derived but also against the infection with other mosquito-borne viruses or other serotypes of the virus from which the vaccine is derived. The NS1 protein can preferably be of any Dengue virus serotype. More preferably the NS1 protein coding sequence is derived from a Dengue virus serotype 2 such as the Dengue virus New Guinea strain („NGC strain", GeneBank accession number AF038403). The terms "subtype" and "serotype" are used interchangeably throughout this description.

The term „part thereof" in the context of the term „NS1 protein or part thereof" refers to an amino acid stretch of the NS1 protein, which is sufficiently long to induce a specific immune response against the NS1

protein from which the „part thereof" is derived. If the Flavivirus is a Dengue virus the amino acid stretch should be an amino acid stretch that provokes an immune response in a vaccinated animal including a human against the NS1 proteins of all Dengue virus serotypes. In the examples section it is shown how the person skilled in the art can determine whether an NS1 protein or part thereof induces an immune response specific for all Dengue virus serotypes. According to a preferred embodiment the Flavivirus DNA sequence encodes the entire NS1 protein. Thus, the term "NS1 protein or part thereof" relates to the entire sequence of naturally occurring NS1 proteins and shorter eptitope stretches that still elicit an immune response.

Moreover the term "NS1 protein" also relates to derivatives of naturally occurring NS1 proteins. Such a derivative may be a protein that has one or more amino acid substitiutions, deletions and/or insertion with respect to the naturally occurring NS1 protein. By way of example such a derivative is a protein that has a homology in the amino acid sequence of at least 50%, preferably of at least 75%, more preferably of at least 90%. Consequently, the term "part thereof" also relates to parts of such a NS1 protein derivative.

In summary one of the most preferred embodiments of the present invention is to use a DNA comprising an expression cassette coding for a mosquito-borne Flavivirus NS1 or part thereof, wherein the Flavivirus is preferably the Dengue virus, in particular Dengue virus serotype 2, and wherein the expression of the NS1 protein or part thereof is controlled by a transcriptional regulatory element. More preferably the DNA according to the present invention encodes the NS1 protein or part thereof as a fusion protein with a glycosylation signal sequence.

The invention further refers to vectors comprising a DNA as described above and to the use of said vectors to induce an immune response according to the present invention. The term „vector" refers to any vectors known to the person skilled in the art. A vector can be a plasmid vector such as pBR322 or a vector of the pUC series. More preferably the vector is a virus vector. In the context of the present invention the term „viral vector" or "virus vector" refers to an infectious virus comprising a viral genome. In this case the DNA of the present invention is to be cloned into the viral genome of the respective viral vector. The recombinant viral genome is then packaged and the thus obtained recombinant vectors can be used for the infection of cells and cell lines, in particular for the infection of living animals including humans. Typical virus vectors that may be used according to the present invention are adenoviral vectors, retroviral vectors or vectors on the basis of the adeno associated virus 2 (AAV2). Most preferred are poxviral vectors. The poxvirus may be preferably a canarypox virus, a fowlpoxvirus or a vaccinia virus. More preferred is modified vaccinia virus Ankara (MVA) (Sutter, G. *et al.* [1994], Vaccine 12: 1032-40). A typical MVA strain is MVA 575 that has been deposited at the European Collection of Animal Cell Cultures under the deposition number ECACC V00120707. Most preferred is MVA-BN or a derivative thereof which has been described in the PCT application WO 02/42480 (PCT/EP01/13628). The content of this application is included in the present application by reference. MVA-BN has been deposited at the European Collection of Animal Cell Cultures with the deposition number ECACC V00083008. By using MVA-BN or a derivative thereof the additional technical problem has been solved to provide a particular safe virus vaccine against Flaviviruses since it has been shown that the MVA-BN virus vector is an extremely attenuated virus. In particular, it has been demonstrated that MVA-BN is more attenuated than the MVA strains known before in the prior art. MVA-BN is derived from Modified Vaccinia Ankara virus and is characterized by the

loss of its capability to reproductively replicate in human cell lines. MVA-BN is safer than any other known vaccinia virus strains due to a lack of replication in humans. In the preferred embodiment the invention concerns as a viral vector containing the DNA as defined above MVA-BN and derivatives of MVA-BN. The features of MVA-BN, the description of biological assays allowing to evaluate whether a MVA strain is MVA-BN or a derivative thereof and methods allowing to obtain MVA-BN or a derivative thereof are disclosed in WO 02/42480.

The term "derivatives" of the virus as deposited under ECACC V00083008, i.e. derivatives of MVA-BN, is used in the present application as defined in WO 02/42480. In the following the features of a derivative of MVA-BN are shortly summarized. For more detailed information regarding the definition of a derivative of MVA-BN and in particular for detailed information regarding the biological assays used to determine whether a MVA virus is a derivative of MVA-BN reference is made to WO 02/42480. Thus, said term refers to vaccinia viruses showing at least one of the following features of the deposited strain MVA-BN but showing differences in one or more parts of its genome. Preferably a derivative has at least two, more preferably at least three, most preferably all of the following four features of MVA-BN:

- capability of reproductive replication in chicken embryo fibroblasts (CEF) and in the baby hamster kidney cell line BHK (ECACC 85011433), but no capability of reproductive replication in the human cell line HaCat (Boukamp *et al.* 1988, J Cell Biol. 106(3): 761-71),
- failure to replicate in vivo,
- induction of a higher immunogenicity compared to the known strain MVA 575 (ECACC V00120707) in a lethal challenge model and/or
- induction of at least substantially the same level of immunity in vaccinia virus prime/ vaccinia virus boost regimes when compared to DNA-prime/ vaccinia virus boost regimes.

In particular a derivative of MVA-BN has essentially the same replication characteristics than MVA-BN. Viruses having the same "replication characteristics" than the deposited virus are viruses that replicate with similar amplification ratios than the deposited strain in CEF cells and the cell lines BHK, HeLa, HaCat and 143B and that show a similar replication in vivo as determined in the AGR129 transgenic mouse model.

The term "not capable of reproductive replication" is used in the present application as defined in WO 02/42480. Thus, a virus that is "not capable of reproductive replication" is a virus that shows an amplification ratio of less than 1 in the human cell line HaCat (Boukamp *et al.* 1988, J Cell Biol. 106(3): 761-71. Preferably, the amplification rate of the virus used as a vector according to the invention is 0.8 or less in the human cell line HaCat. The "amplification ratio" of a virus is the ratio of virus produced from an infected cell (Output) to the amount originally used to infect the cells in the first place (Input) ("amplification ratio"). A ratio of "1" between Output and Input defines an amplification status wherein the amount of virus produced from the infected cells is the same as the amount initially used to infect the cells.

In the context of the definition of MVA-BN and its derivatives the term "failure to replicate in vivo" is used in the present application as defined in WO 02/42480. Thus, said term refers to viruses that do not replicate in humans and in the mice model as explained in WO 02/42480. The mice used in WO 02/42480 are incapable of producing mature B- and T-cells (AGR 129 mice). In particular MVA-BN and its derivatives do not kill AGR129 mice within a time period of at least 45 days, more preferably within at least 60 days, most preferably within 90 days after the infection of the mice with 10^7 pfu virus administered intra peritonealy. Preferably, the viruses that show "failure to replicate in vivo" are further characterized in that no virus can be recovered from organs or tissues of

the AGR129 mice 45 days, preferably 60 days and most preferably 90 days after the infection of the mice with 10^7 pfu virus administered intra peritonealy.

MVA-BN and its derivatives are preferably characterized by a higher immunogenicity compared to the known strain MVA 575 as determined in a lethal challenge mouse model as explained in WO 02/42480. In such a model unvaccinated mice die after the infection with replication competent vaccinia strains such as the Western Reserve strain L929 TK+ or IHD-J. The infection with replication competent vaccinia viruses is referred to as "challenge" in the context of description of the lethal challenge model. Four days after the challenge the mice are usually killed and the viral titer in the ovaries is determined by standard plaque assays using VERO cells. The viral titer is determined for unvaccinated mice and for mice vaccinated with MVA-BN and its derivatives. More specifically MVA-BN and its derivatives are characterized in that in this test after the vaccination with 10^2 $TCID_{50}$/ml virus the ovary virus titers are reduced by at least 70%, preferably by at least 80%, more preferably by at least 90% compared to unvaccinated mice.

MVA-BN or its derivatives are preferably characterized by inducing at least substantially the same level of immunity in vaccinia virus prime/ vaccinia virus boost regimes when compared to DNA-prime/ vaccinia virus boost regimes. A vaccinia virus is regarded as inducing at least substantially the same level of immunity in vaccinia virus prime/ vaccinia virus boost regimes when compared to DNA-prime/ vaccinia virus boost regimes if the CTL response as measured in one of the „assay 1" and „assay 2" as disclosed in WO 02/42480, preferably in both assays, is at least substantially the same in vaccinia virus prime/ vaccinia virus boost regimes when compared to DNA-prime/ vaccinia virus boost regimes. More preferably the CTL response after vaccinia virus prime/vaccinia

virus boost administration is higher in at least one of the assays, when compared to DNA-prime/vaccinia virus boost regimes. Most preferably the CTL response is higher in both assays.

WO 02/42480 discloses how Vaccinia viruses are obtained having the properties of MVA-BN and its derivatives as defined above.

Methods to insert the DNA as defined above into poxviral DNA and methods to obtain recombinant poxviruses are known to the person skilled in the art. In a recombinant vaccinia virus the expression of the DNA according to the present invention is preferably, but not exclusively, under the transcriptional control of a poxvirus promoter, more preferably of a vaccinia virus promoter. The insertion of the DNA according to the present invention is preferably into a non-essential region of the virus genome. In another preferred embodiment of the invention, the heterologous nucleic acid sequence is inserted at a naturally occurring deletion site of the MVA genome (disclosed in PCT/EP96/02926).

In summary it is one of the most preferred embodiments of the present invention to provide a vector comprising the DNA as defined above, wherein the vector is MVA-BN or a derivative thereof and wherein the DNA comprises an expression cassette coding for a Flavivirus NS1 protein or part thereof, wherein the Flavivirus is preferably a Dengue virus, more preferably Dengue virus serotype 2.

In a preferred embodiment the invention relates to the usefulness of the NS1 protein or part thereof encoded by a DNA according to the present invention or by a vector according to the present invention for vaccination against several flaviviruses or flavivirus serotypes. For the definition of the NS1 protein or part thereof according to the present invention reference is made to the above parts of the description where

the DNA encoding NS1 has been defined by the product expressed from said DNA. The following summary regarding the protein according to the present invention is therefore not to be regarded as a limitation of the invention. In summary the NS1 protein can be an isolated NS1 protein or part thereof encoded by any Flavivirus. The NS 1 protein or part thereof is preferably derived from a Dengue virus, most preferably from Dengue virus serotype 2. The protein may only comprise the amino acid sequence of a viral NS1 protein or part thereof. In a preferred embodiment the NS1 protein may contain additional amino acids that are required for an effective expression of the protein. Examples for such amino acids/amino acid sequences are shown above and include the methionine at the N-terminus of the protein encoded by an added ATG codon and an amino acid sequence derived from the C-terminal end of the E-protein acting as a signal sequence for glycosylation of the NS1 protein or part thereof. Other signal sequences are also within the scope of the present invention. In an alternative embodiment the NS1 amino acid sequence or a part thereof can be fused to other proteins/peptides. Examples for fusion partners are sequences allowing the identification of the protein such as tags or other flaviviral proteins or parts thereof.

In a preferred embodiment the present invention concerns the DNA, the vector or the NS1 protein or part thereof according to the present invention as a vaccine, in particular as a vaccine against several flaviviruses or flavivirus serotypes. A „vaccine" is a compound, i.e. a DNA, a protein, a vector or a virus that induces a specific immune response.

According to one alternative of this embodiment the „vaccine" according to the present invention is based on a Dengue virus NS1 protein or a part thereof which induces an immune response against the NS 1 proteins of all Dengue virus serotypes. In particular it has been shown that the NS1 protein of one Dengue virus serotype, in particular serotype

2, induces an immune response against the NS1 proteins of at least two, preferably at least three, most preferably all Dengue virus serotypes and preferably also against at least one other mosquito-borne Flavivirus..

As explained above the inventors of the present invention have found that the NS1 protein or part thereof according to the present invention of one Flavivirus induces an immune response against the NS1 protein of other Flaviviruses. As pointed out above the "Flavivirus" is preferably a mosquito-borne Flavivirus. In other words the inventors of the present invention have found that in an alternative embodiment the NS1 protein or part thereof according to the present invention of one mosquito-borne Flavivirus induces an immune response against the NS1 protein of the mosquito-borne Flavivirus from which the vaccine is derived and also against other mosquito-borne Flaviviruses. Thus, the vaccine derived from a mosquito-borne Flavivirus is useful as vaccine against one or more mosquito-borne flaviviruses. The term "vector derived from a Flavivirus" or similar terms in the context of the present description means that a vector as defined above (e.g. a poxvirus vector or a plasmid) contains a DNA as defined above. Thus, this term refers to the vector insert and not the vector backbone. An example for a "vector derived from a Flavivirus" is a poxvirus vector, such as MVA, comprising an expression cassette comprising a poxvirus promoter, a sequence encoding a Flavivirus NS1 protein or part thereof, wherein the sequence coding for the Flavivirus NS1 protein or part thereof is preceded by an ATG codon and a sequence ecoding a glycosylation signal sequence and wherein the coding sequence is terminated by a stop codon of translation.

Thus the vaccination with the DNA, the vector or the NS1 protein or part thereof is useful as a single subunit vaccine against a broad range of Flaviviruses or at least Flavivirus serotypes. The DNA or vector encoding the NS1 protein or part thereof from one Flavivirus or Flavivirus serotype

or the NS 1 protein or part thereof from said Flavivirus or serotype can thus be used as a vaccine for vaccination against other Flaviviruses and Flavivirus serotypes, respectively. For example a vaccine derived from a Dengue virus serotype 2 can be used as a vaccine against one, two or all of the serotypes 1, 3 and 4, as well as vaccine against serotype 2. It may further be useful to protect an individual against other Flaviviruses such as the West Nile Virus, the Japanese encephalitis virus and the Yellow fever virus.

In a preferred embodiment the DNA according to the present invention is used as a vaccine. It is known by the person skilled in the art that the administration of naked DNA harboring a eukaryotic expression cassette as in the present invention, in particular the intramuscular injection of DNA leads to the expression of the protein encoded by the expression cassette. The protein is exposed to the immune system and a specific immune response is raised.

In an alternative embodiment the vaccination is made by administering a vector according to the present invention, in particular a viral vector, more preferably a poxvirus vector, most preferably a vaccinia virus vector, e.g. a MVA vector.

For the preparation of vaccinia virus based vaccine, the virus according to the invention, in particular MVA-BN and its derivatives, is converted into a physiologically acceptable form. This can be done based on the experience in the preparation of poxvirus vaccines used for vaccination against smallpox (as described by Stickl, H. *et al.* [1974] Dtsch. med. Wschr. 99, 2386-2392). For example, the purified virus is stored at –80°C with a titer of $5x10^8$ $TCID_{50}$/ml formulated in about 10mM Tris, 140 mM NaCl pH 7.4. For the preparation of vaccine shots, e.g., 10^2-10^9 particles of the virus are lyophilized in 100 ml of

phosphate-buffered saline (PBS) in the presence of 2% peptone and 1% human albumin in an ampoule, preferably a glass ampoule.

It is particularly preferred that the vaccinia virus based vaccine, in particular a MVA-BN based vaccine, used for vaccination is stored in a freeze-dried state. It is shown in the example section that the immune reaction as well as the percentage of cross reaction of the immune response induced by the NS1 protein of one flavivirus to the NS1 protein of different flaviviruses and flavivirus serotypes, respectively, is particularly high if the virus used for vaccination was stored as freeze dried virus. Thus, the vaccine shots preferably can be produced by stepwise freeze-drying of the virus in a formulation. This formulation can contain additional additives such as mannitol, dextran, sugar, glycine, lactose or polyvinylpyrrolidone or other additives such as antioxidants or inert gas, stabilizers or recombinant proteins (e.g. human serum albumin) suitable for *in vivo* administration. An typical virus containing formulation suitable for freeze-drying comprises 10 mM Tris-buffer, 140 mM NaCl, 18.9 g/l Dextran (MW 36000 - 40000), 45 g/l Sucrose, 0.108 g/l L-glutamic acid mono potassium salt monohydrate pH 7.4. After freeze-drying the glass ampoule is then sealed and can be stored between 4°C and room temperature for several months. However, as long as no need exists the ampoule is stored preferably at temperatures below -20°C. For vaccination the lyophilisate can be dissolved in 0.1 to 0.5 ml of an aqueous solution, such a water, physiological saline or Tris buffer, and administered either systemically or locally, i.e. by parenterally, intramuscularly or any other path of administration know to the skilled practitioner. The mode of administration, the dose and the number of administrations can be optimized by those skilled in the art in a known manner. Most preferred for poxvirus vectors is subcutaneous or intramuscular administration. Most preferably the vaccination is done by administration of two vaccine shots in an interval of e.g. 3 to 5 weeks.

If the vaccine is a MVA-BN vector or derivative thereof comprising a DNA according to the present invention a particular embodiment of the present invention concerns a kit for vaccination comprising a MVA-BN virus vector according to the present invention for the first vaccination („priming") in a first vial/container and for a second vaccination („boosting") in a second vial/container.

If the vaccine is a MVA-BN vector or derivative thereof comprising a DNA as defined above a particular embodiment of the present invention concerns the administration of the vaccine in therapeutically effective amounts in a first inoculation ("priming inoculation") and in a second inoculation ("boosting inoculation"). The interval between the priming inoculation and the boosting inoculation is e.g. 2 to 12 weeks, preferably e.g. 3-6 weeks, more preferably e.g. about 3 weeks. The virus amount used for vaccination shout be at least 1 x 10^2 $TCID_{50}$, preferably e.g. 1 x 10^7 $TCID_{50}$ to 1 x 10^9 $TCID_{50}$. Moreover, a particular embodiment of the present invention concerns a kit for vaccination comprising a MVA-BN virus vector as defined above for the first vaccination („priming") in a first vial/container and for a second vaccination („boosting") in a second vial/container.

Thus, the invention concerns in the vaccine embodiments a vaccine comprising a DNA, a vector or a NS1 protein or part thereof as defined above and the use of said DNA, vector or protein for the preparation of a vaccine. According to a preferred embodiment the invention concerns the use of said DNA, vector or protein for the preparation of a vaccine wherein the NS1 protein or part thereof, the NS1 protein or part thereof encoded by the DNA or the vector is from one Dengue virus serotype and wherein the DNA, the vector or the NS 1 protein or part thereof is used as a

vaccine against two, three or all Dengue virus serotypes. Most preferably the Dengue virus serotype is serotype 2.

The invention further relates to a method for the treatment or prevention of a Flavivirus infection comprising inoculating an animal, including a human, in need thereof with a DNA as above, a vector as above or a NS1 protein or part thereof as above. In particular the invention relates to a method as above, wherein the NS1 protein or part thereof or the NS1 protein or part thereof encoded by the DNA or the vector is from one Dengue virus serotype and wherein the DNA, the vector or the NS 1 protein or part thereof is used as a vaccine against two, three or all Dengue virus serotypes

Summary of the Invention

The invention relates in particular to the following, alone or in combination:

Use of

- a nucleic acid comprising an expression cassette comprising a transcriptional regulatory element and a sequence which codes at least for the NS1 protein or a part thereof of a mosquito-born flavivirus,
- a vector comprising said nucleic acid and/or
- a NS1 protein or part thereof of said flavivirus

for the preparation of a vaccine against the mosquito-borne Flavivirus from which the nucleic acid or the NS 1 protein or part thereof is derived and against at least one other mosquito-borne Flavivirus.

Use as above, wherein the mosquito-born Flavivirus from which the nucleic acid or the NS1 protein or part thereof is derived is a Dengue virus.

Use of a

- nucleic acid comprising an expression cassette comprising a transcriptional regulatory element and a sequence which codes at least for the NS1 protein or a part thereof of a Dengue virus serotype,
- vector comprising said nucleic acid and/or
- NS1 protein or part thereof of said Dengue virus serotype

for the preparation of a vaccine against all Dengue virus serotypes and optionally against at least one other mosquito-borne Flavivirus.

Use as above, wherein the Dengue virus from which the nucleic acid or the NS1 protein or part thereof is derived is Dengue virus serotype 2.

Use as above, wherein the sequence coding for the NS1 protein or part thereof of the mosquito-borne flavivirus or of the Dengue virus serotype is preceded by an ATG codon and a sequence encoding a glycosylation signal sequence and wherein the coding sequence is terminated by a stop codon of translation.

Use as above, wherein the other mosquito-borne Flavivirus is selected from the West Nile virus, the Yellow fever virus and the Japanese Enzephalitis virus.

Use as above, wherein the vector is a poxvirus vector.

Use as above, wherein the poxvirus vector is a Modified Vaccinia Virus Ankara (MVA) strain; in particular MVA-BN deposited at the European Collection of Cell Cultures under number V00083008 or a derivative thereof.

Use as above, wherein the poxvirus vector is freeze-dried and is reconstituted in a pharmaceutically acceptable diluent prior to administration.

Use as above, wherein the transcriptional regulatory element is a poxvirus promoter.

Use as above, wherein the vaccine is administered in therapeutically effective amounts in a first inoculation ("priming inoculation") and in a second inoculation ("boosting inoculation")

Method for the treatment or prevention of a flavivirus infections comprising inoculating an animal, including a human, in need thereof with

- a nucleic acid comprising an expression cassette comprising a transcriptional regulatory element and a sequence which codes at least for the NS1 protein or a part thereof of a mosquito-born flavivirus,
- a vector comprising said nucleic acid and/or
- a NS1 protein or part of said flavivirus,

wherein the Flavivirus infection is an infection by the mosquito-borne Flavivirus from which the nucleic acid, or the NS 1 protein or part thereof is derived and/or an infection by another mosquito-borne Flavivirus.

Method as above, wherein the mosquito-borne Flavivirus from which the nucleic acid or NS1 protein or part thereof is derived is a Dengue virus.

Method for the treatment or prevention of a Flavivirus infection comprising inoculating an animal, including a human, in need thereof with

- a nucleic acid comprising an expression cassette comprising a transcriptional regulatory element and a sequence which codes at least for the NS1 protein or a part thereof of a Dengue virus serotype,
- a vector comprising said nucleic acid and/or
- a NS1 protein or part thereof of said Dengue virus serotype,

wherein the Flavivirus infection is an infection by the Dengue virus serotype from which the nucleic acid, or the NS 1 protein or part thereof is derived and/or an infection by another Dengue virus serotypes and/or an infection by other mosquito-borne Flaviviruses.

Method as above, wherein the Dengue virus from which the DNA or the protein or part thereof is derived is Dengue virus serotype 2.

Method as above, wherein the sequence coding for the NS1 protein or part thereof of the mosquito-borne flavivirus or of the Dengue virus serotype is preceded by an ATG codon and a sequence encoding a

glycosylation signal sequence and wherein the coding sequence is terminated by a stop codon of translation.

Method as above, wherein the vector is a poxvirus vector.

Method as above, wherein the poxvirus vector is a Modified Vaccinia Virus Ankara (MVA) strain

Method as above, wherein the MVA strain is MVA-BN deposited at the European Collection of Cell Cultures under number V00083008 or a derivative thereof.

Method as above, wherein the poxvirus vector is freeze-dried and is reconstituted in a pharmaceutically acceptable diluent prior to administration.

Method as above, wherein the transcriptional regulatory element is a poxvirus promoter.

Method as above, wherein the poxvirus vector or the pharmaceutical composition is administered in therapeutically effective amounts in a first inoculation ("priming inoculation") and in a second inoculation ("boosting inoculation")

Poxvirus vector harboring a DNA comprising an expression cassette comprising a transcriptional regulatory element and a sequence which codes at least for a Flavivirus NS1 protein or a part thereof, wherein the poxvirus is Modified Vaccinia Virus Ankara (MVA) strain BN deposited at the European Collection of Cell Cultures under number V00083008 or a derivative thereof.

Poxvirus vector as above, wherein the Flavivirus is a mosquito-borne Flavivirus, in particular a Dengue virus.

Poxvirus vector as above, wherein the Dengue virus is Dengue virus serotype 2.

Poxvirus vector as above, wherein the sequence coding for the Flavivirus NS1 protein or part thereof is preceded by an ATG codon and a sequence encoding a glycosylation signal sequence and wherein the coding sequence is terminated by a stop codon of translation.

Poxvirus vector as above, wherein the transcriptional regulatory element is a poxvirus promoter.

Poxvirus vector as above, wherein the poxvirus vector is freeze-dried.

Poxvirus vector as above as a vaccine.

Pharmaceutical composition comprising a poxvirus vector as above and a pharmaceutically acceptable carrier, diluent and/or additive.

Poxvirus vector as above or pharmaceutical composition as above for the treatment and/or prevention of a flavivirus infection, wherein the poxvirus vector or the pharmaceutical composition is administered in therapeutically effective amounts in a first inoculation ("priming inoculation") and in a second inoculation ("boosting inoculation")

Method for the treatment or prevention of a flavivirus infection comprising inoculating an animal, including a human, in need thereof with a vector as above or with the pharmaceutical composition as above.

Cell, preferably a human cell, comprising a poxvirus vector as above.

Use of the poxvirus vector as above for the preparation of a vaccine to treat or to prevent a Flavivirus infection.

Kit for prime/boost immunization comprising a poxvirus vector as above or a pharmaceutical composition as above for a first inoculation ("priming inoculation") in a first vial/container and for a second inoculation ("boosting inoculation") in a second vial/container.

Short Description of the Figures

Figure 1A: The dengue NGC strain "signal sequence+NS1" cDNA protein coding sequence of the construct used as an example in this invention. The start of the NS1 gene in the natural context is indicated by an arrow. Important features are the addition of an ATG start codon and a stop codon (in this example "TAG"). Nucleotide sequence numbers refer to position in the NGC strain genome (Genbank accession number AF038403). The nucleotide and amino acid sequence in Figure 1A corresponds to SEQ:ID No. 5. The amino acid is separately shown as SEQ:ID No. 6.

Figure 1B: Diagram of plasmid pAF7NS1 containing the dengue NGC strain "signal sequence+NS1" protein coding sequence.

Figure 1C: Nucleotide sequence of the NS1 cassette within plasmid pAF7 showing the primer binding sites for PCR amplification of this cassette with oBN345 and oBN338. The nucleotide and amino acid sequence in Figure 1C corresponds to SEQ:ID No. 7. The amino acid is separately shown as SEQ:ID No. 8.

Figure 1D: top: Kyte-Doolittle hydrophicity plot of dengue NGC strain NS1 amino acid sequence (amino acid 776 to 1127 of dengue NCG polyprotein Genbank Accession AF038403). Values above zero = hydrophobic. bottom: Kyte-Doolittle hydrophicity plot of dengue NGC strain NS1 amino acid sequence containing a signal sequence derived from the last 28 amino acids of C-terminal of E protein (amino acid 748 to 775). The total amino acid sequence represents amino acid 748 to 1127 of the dengue NCG polyprotein (Genbank Accession AF038403) which for this strain starts with an "ATG" start codon but lacks a stop codon. Sig = Signal sequence. Values above zero = hydrophobic

Figure 2: Nucleotide sequence of the "poxvirus promoter+signal sequence+NS1" expression cassette. The nucleotide and amino acid sequence in Figure 2 corresponds to SEQ:ID No. 9. The amino acid is separately shown as SEQ:ID No. 10. Briefly, the minimal poxvirus early/late promoter element controls the expression of the NS1 protein of Dengue virus serotype 2, wherein the N-terminus of the NS1 protein is fused to the 28 C-terminal aminoacids of the E-protein. The translation is terminated at an TAG stop codon that has been inserted into the nucleic acid sequence.

Figure 3A: Cloning of NS1 expression cassette into the blunt ended Xho I site (blunt end cloning) of pBNX07 to produce the clone pBN41. PPr = poxvirus promoter, D2F1 = flank 1 of deletion site 2, NPT II = neomycin resistance gene, IRES = Internal Ribosome Binding Site, EGFP = Enhanced Green Fluorescence Protein, NS1 (in pBN41) = signal sequence+NS1, D2F2 = Flank 2 of deletion site 2, Sig = signal sequence. AmpR = Ampicillin resistance gene.

Figure 3B: Hind III map of MVA (Genbank U94848) showing the location of the six deletion sites of MVA (-J- = junction of the deletion site). The "PPr+NPT II+IRES+EGFP+PPr+NS1" cassette was inserted into deletion 2 site of MVA. PPr = poxvirus promoter, NPT II = neomycin resistance gene (protein coding sequence), IRES = Internal Ribosome Binding Site and NS1 = signal sequence plus NS1 protein coding sequence of dengue 2 NGC strain.

Figure 4: Plot of the ELISA absorbance readings of the post-immunized sera titrations for all three rabbits.

Figure 5: Elisa cross reactivity studies. The cross reactivity of a rabbit serum of day 38 (upper part) and day 66 (lower part) with lysates of cells infected with DENV-1, DENV-3, DENV-4, JEV and WNV was tested in an ELISA-assay.

Examples

The following examples will further illustrate the present invention. It will be well understood by a person skilled in the art that the examples may not be interpreted in a way that limits the applicability of the technology provided by the present invention to these examples.

Example 1: Construction of mBN07

1. Details of NS1 antigen (Figure 1)

The example refers to NS1 of serotype 2 derived from the New Guinea C strain - NGC strain (example: Genbank sequence AF038403). Since the NS1 protein of the Flaviviruses is produced as part of a polyprotein precursor the NS1 gene in the corresponding DNA is not preceded by a „ATG" start codon.

Therefore, a cDNA sequence coding for the NS1 protein must require the addition of a "ATG" start codon. This is then followed by the addition of a signal sequence so that the newly synthesized NS1 protein becomes glycosylated in the endoplasmic reticulum. Finally, the protein-coding cassette needs a stop codon and in this example TAG was added to the 3' terminal end of the protein coding cDNA sequence. In the example used in this invention the "ATG+signal sequence" element was derived from the hydrophobic C-terminal end of the E protein (the last 28 amino acids, which for NGC strains starts with the amino acid M (ATG)).

Fig 1A shows the exact signal sequence plus NS1 sequence used as the example for this invention (see also SEQ:ID 5 and 6). The "signal sequence+NS1" nucleotide coding sequence was obtained by RT-PCR amplification from dengue NGC genomic RNA using the following primers:

D2NS1-1up : 5'-ACA*AGATCT*GGAATGAATTCACGTAGCACCTCA-3' (SEQ:ID No. 4)
In italics: Bgl II restriction endonuclease recognition site.
Underlined is the start codon.

D2NS1-2down: 5'-AAT*AGATCT*CTACTAGGCTGTGACCAAGGAGTT-3' (SEQ:ID No. 3)
In italics: Bgl II restriction endonuclease recognition site.
Underlined is the stop codon.

The RT-PCR amplification was carried out using the Titan One Tube RT-PCR kit from Roche Molecular Biochemical (Catalog number 1-939-823) following the instructions recommended by the manufacturer. However, essentially any commercial or non-commercial RT-PCR kit can be used instead.

The RT-PCR product can then be cloned into the BamHI site of any multiple cloning site present in many of the commercial bacterial cloning plasmids available but in this example it was cloned into pAF7 to give rise to clone to pAF7D2NS1 - see fig 1B and 1C for sequence details for pAF7D2NS1. Fig 1D shows the hydrophobicity plots of NS1 amino acid sequence and NS1 containing the added signal sequence from the C-terminal amino acid coding sequence of E protein. The short N-terminal hydrophobic domain is indicative of a signal sequence.

2. **Details of NS1 expression cassette** (Figure 2)

To express this "signal sequence+NS1" from a poxvirus vector such as canarypox, fowlpox, vaccinia or MVA, a poxvirus promoter needs to be added to the 5' end of this cDNA. Poly adenylation signal sequences are not required as all poxvirus synthesized RNAs are polyadenylated by a

virally encoded enzyme that requires no polyA addition signal sequence for carrying out this function. Any poxvirus promoter can be used for the expression of this cassette. Figure 2 and SEQ:ID No. 9 and 10 show the nucleotide sequence of the "poxvirus promoter+signal sequence+NS1" cassette used as the example in this invention.

For the example used in this invention the "signal sequence+NS1" was further PCR amplified from the NS1 plasmid clone using the primers oBN338 and oBN345. oBN345 primer contains a nucleotide sequence of a poxvirus minimal promoter element 5' to the target sequence within the cloning plasmid. Plasmid target sequence for oBN345 primer binding was approximately 40 nucleotides upstream of the signal sequence start codon. This was to ensure the RNA transcript contain a stretch of non-protein coding sequence before the signal sequence ATG start codon.

PCR primers with Ps promoter:

oBN338: 5'-TTGTTAGCAGCCGGATCGTAGACTTAATTA (30mer) (SEQ:ID No. 1)

oBN345:

5'-CAAAAAATTGAAATTTTATTTTTTTTTTTTGGAATATAAATAAAA ACACGATAATACCATGG -3'

(SEQ:ID No. 2)

(Underlined nucleotides present the poxvirus minimal promoter sequence.)

Annealing temperature for the PCR amplification reaction for the first five cycles was calculated from the nucleotide sequence that binds to the homologous sequence in the cloning vector of oBN345.

3. Integration of NS1 expression cassette into MVA (Fig 3)

The PCR amplification product was blunt end cloned into the cut and blunt ended Xho I site of plasmid pBNX07 (see Fig 3a) to form plasmid pBN41 (see Fig 3a). pBN41 is the vector for integrating the "pox promoter+signal sequence+NS1" cassette into deletion site 2 of MVA by homologous recombination.

The essential features of pBN41 (see Fig3a) are as follows:

- Plasmid backbone is pBluescript SK-plus from Stratagene (Genbank VB0078)
- D2F1: Deletion 2 flank 1 homologous recombination arm. This represents the nucleotide sequence from 20117 to 20717 of the MVA Genbank sequence U94848.
- PPr: Poxvirus promoter.
- NPT II: Neomycin phoshotransferase protein coding sequence (protein coding sequence of Genbank V00618).
- IRES: Internal Ribosome Entry Sequence from encephalomyocarditis virus (Jang et al., 1989, Genbank M16802).
- EGFP: Enhanced Green Fluorescence Protein coding sequence (protein coding sequence - nucleotide 675 to nucleotide 1394 of Genbank sequence U57609)
- NS1: "signal sequence+NS1" protein coding sequence from dengue NGC strain.
- D2F2: Deletion 2 Flank 2 homologous recombination arm. This represents the nucleotide sequence from 20719 to 21343 of the MVA Genbank sequence U94848.
- AmpR: Ampicillin resistance gene of pBluescript

3.1 Insertion of dengue "pox promoter+signal sequence+NS1" into deletion site of MVA by homologous recombination

3.1.1 Integration into MVA Genome by Homologous Recombination

The above integration vector pBN41 is used to integrate the dengue NS1 expression cassette plus also the reporter cassette (Pox promoter+NPT II-IRES-EGFP) into the MVA genome by homologous recombination between flank 1 and flank 2 arms of pBN41 and the homologous target sequences within the MVA genome. This is achieved by transfecting the linearized integration vector into chicken embryo fibroblast (CEF) cells previously infected with MVA at low multiplicity of infection (MOI, for example, 0.01 infectious units per cell). At 48 hours post infection or when the infection had reached confluency a viral extract is prepared and stored at –20°C ready for selection and clone purification of desired recombinant MVA (rMVA).

3.1.2 Selection of rMVA and Clone Purification

The elimination of non-recombinant MVA (empty vector virus) and the amplification of rMVA is achieved by infection of confluent chicken embryo fibroblast (CEF) cells at low MOI in the presence of G418 (amount of G418 has to be optimize to determine the highest dose that dose not kill the CEF cells). Any virus that does not contain and integrated NPT II gene will not replicate in the presence of G418 added to the cell maintenance medium. G418 inhibits DNA replication but since the CEF cells will be in the stationary non-replicating state they will not be affected by the action of G418. CEF cells infected with rMVAs can be visualized under a fluorescence microscope due to the expression of the enhanced fluorescent green protein.

Viral extracts from the homologous recombination step must be serially diluted and used to infect fresh CEF cells in the presence of G418 and

overlaid with low-melting point agarose. After 2 days of infection, the agarose-infected plates are observed under a fluorescent microscope for single foci of green infected cells. These cells are marked and agarose plugs containing the infected foci of cells are taken and placed into 1.5 ml microcentrifugation tubes containing sterile cell maintenance medium. Virus is released from the agarose plug by freeze-thawing the tube three times at –20°C.

The best clone or clones are further clone purified under agarose until by PCR analysis there is no signs of empty vector contamination (3 to 30 rounds of clone purification). These clones are then amplified for further stringent testing for correct insertion configuration, sequence verification of promoter-foreign gene cassette and expression analysis by RT-PCR. After these analysis only one clone was further amplified under G418 selection to prepare a master stock for further characterization and immunogenicity studies.

The recombinant MVA with the inserted dengue NS1 expression cassette described in this invention was named mBN07. Fig3b shows the configuration of inserted foreign sequence in mBN07.

4. Expression of authentic NS1 by MVA

The expression of the NS1 protein from the recombinant MVA, mBN07, was verified by standard western blot analysis under non-denaturing conditions. More particularly, NS1 expression was analyzed after purified mBN07 was used to infect mammalian tissue culture cells, for example BHK-21 cells, at an MOI of 1.0 infectious unit per cell. Crude protein extracts were prepared from these infected cells 24-30 hours after infection where portions of these extract were mixed with SDS-PAGE gel

loading buffer containing 2-mercaptoethanol (2-ME) or not containing 2-ME. These samples plus protein extract from cells (mosquito cell line) infected with dengue NGC strain as a positive control were electrophoretically separated in a SDS-PAGE gel and then blotted onto nitrocellulose membrane. The membrane was probed with an anti-dengue NS1 monoclonal antibody.

It was shown that NS1 expressed by mBN07 is recognized by the anti-dengue NS1 monoclonal antibody and forms the correct dimeric form similar to NS1 from dengue infected cells (compare unboiled mBN07 without 2-ME lane with unboiled DEN2 without 2-ME lane). Moreover it was shown that the dimeric form resolves out to the monomeric forms under denaturing conditions (see boiled mBN07 with 2-ME lane).

The NS1 expressed in cells infected with mBN07 was also recognized by pooled convalescent patients' sera and with monoclonal antibodies that cross-react to NS1 of all four serotypes of dengue in western blot analysis. This demonstrates that NS1 expressed by mBN07 is immunogenic.

Throughout the example section mBN07 (sometimes also termed BN07) is either stored in a liquid state (optionally frozen) or in a freeze-dried state. To obtain a freeze-dried virus a virus containing solution is prepared that comprises 10 mM Tris-buffer, 140 mM NaCl, 18.9 g/l Dextran (MW 36000 - 40000), 45 g/l sucrose, 0.108 g/l L-glutamic acid mono potassium salt monohydrate pH 7.4. Said formulation is then freeze-dried. For reconstitution water is added to the freeze-dried preparation.

Example 2: Cross immunogencity of NS1 expressed by mBN07 to NS1 of Dengue viruses other than serotype 2 and to the NS1 of Japanese encephalitis virus (JEV) and West nile virus (WNV)

1. NS1 expressed from mBN07: reactivity to patient convalescent sera

Tested was the possibility that NS1 expressed by mBN07 is recognized by convalescent patient sera from individuals who have evidence of previous dengue virus infection. Serum from 68 individuals who have antibodies against dengue virus envelope protein (by immunoblotting against authentic antigens prepared from Dengue serotypes 1 to 4) were selected for testing against immunoblot strips prepared from mBN07 infected cell extracts and MVA-GFP infected cell extracts as control. The antigen-containing cell lysates were treated with sample buffer without 2 mercaptoethanol and were not heated. Of the 68 individual sera tested, 62 (91.2%) reacted with BN07 NS1 expressed by mBN07 in immunoblots. These sera were further analyzed for reactivity to NS1 of all 4 dengue virus serotypes as well as Japanese encephalitis virus (JEV). The results are shown in Table 1. Fifty-four of the sera reacted with NS1 of all dengue virus serotypes and Japanese Encephalitis Virus (JEV), and 53 of these 54 sera (98.2%) also reacted with NS1 expressed from mBN07. Seven (7) sera were specific for NS1 of at least one dengue virus serotype and did not react with NS1 of JEV. All these 7 sera also reacted with NS1 expressed by mBN07. Another 7 sera reacted only with NS1 of JEV and not NS1 of any dengue virus serotype, yet 2 (28.6%) of these JEV-specific sera also reacted with NS1 expressed by mBN07.

Table 1:

	BN07 NS1 NEGATIVE	BN07 NS1 POSITIVE

AUTHENTIC DEN NS1 POSITIVE	0% (0/7)	100% (7/7)
AUTHENTIC DEN & JEV NS1 POSITIVE	1.85% (1/54)	98.15% (53/54)
AUTHENTIC JEV NS1 POSITIVE	71.43% (5/7)	28.57% (2/7)

Comparison of antiserum reactions against authentic NS1 and NS1 expressed by mBN07. In brackets: Number of samples tested positive/Total number of samples tested. DEN = dengue, JEV = Japanese encephalitis virus.

The same 68 sera were also analyzed by reactivity against premembrane proteins. In the experience of the inventors, antibodies against premembrane are far more specific than antibodies against NS1 or E. Thus patients who have been infected with dengue will produce antibodies which recognize dengue virus premembrane and not JEV premembrane and vice versa. An analysis along these lines will provide a better prediction of the history of infection of individuals. Table 2 shows that sera from 22 patients reacted with authentic dengue premembrane protein alone thus suggesting that these 22 patients have been exposed to dengue virus only and not to JEV. All these 22 sera reacted with NS1 expressed by mBN07. Another 22 patients had evidence of previous infection with both dengue and JEV, and again all 22 sera also reacted with NS1 expressed by mBN07. In this series there were also 21 patients who had evidence of previous infection with JEV only (even though these sera had cross-reactive antibodies against dengue E). Interestingly, 17 of the 21 (82%) JEV responders reacted with NS1 expressed by mBN07. There were only 3 sera in the whole set that did not react with premembrane protein of either dengue or JEV and only 1 of these reacted with NS1 expressed by mBN07. The most likely reason for this is that the antibody titer is too low to be detected by immunoblotting.

Table 2:

	BN07 NS1 NEGATIVE	BN07 NS1 POSITIVE
AUTHENTIC DEN prM POSITIVE	0% (0/22)	100% (22/22)
AUTHENTIC DEN & JEV prM POSITIVE	0% (0/22)	100% (22/22)
AUTHENTIC JEV prM POSITIVE	19.0% (4/21)	81.0% (17/21)
PrM NEGATIVE	66.7% (2/3)	33.3% (1/3)

Comparison of antiserum reactions against authentic premembrane and BN07 NS1. In brackets: Number of samples tested positive/Total number of samples tested. DEN = dengue, JEV = Japanese encephalitis virus.

The data in table 2 also clearly shows that of the 6 sera which did not react with NS1 expressed by mBN07, 4 were from individuals who had been previously infected with JEV and not dengue. The remaining 2 had no detectable antibodies to premembrane protein of either dengue or JEV and were likely to have been of a low titer.

2. mBN07 vaccination of Rabbits and testing of post-immunization sera against dengue virus and Japanese encephalitis virus immunoblots and ELISA assays

Three specific pathogen-free rabbits were immunized by subcutaneous route according to the vaccination schedule as shown below. Each rabbit was vaccinated with one vial of freeze-dried vaccine (1x10e8 TCID50 BN07 freeze-dried vaccine) reconstituted to 1ml with sterile water on day 0 and then again on day 28. Blood samples were taken prior to first vaccination (prebleed) and again 10 days after second vaccination.

Day 0 = prebleed followed by 1st vaccination

Day 28 = 2nd vaccination

Day 38 = blood sampling

Day 56 =3rd vaccination

Day 66 =blood sampling

Day 112 =50ml blood withdrawal from each rabbit

2.1 Testing of prebleed and post-immunization sera against dengue serotype 2 immunoblots

Dengue 2 virus antigens and antigens of uninfected C6/36 cells were separated by SDS PAGE under non-denaturing conditions. For immunoblot assays serum of day 38 (diluted 1:200) was used.
The results clearly demonstrated that upon vaccination with mBN07 all three rabbit produced anti-NS1 antibodies of high titres that cross react with authentic NS1 produced from a dengue serotype 2 infection of tissue culture mosquito cells. Serum taken before vaccination did not react to any of the dengue protein on the immunoblots.

Post-immunized serum of day 38 was titrated at 1:1000, 1:2000, 1:4000, $1:10^{-4}$, $1:10^{-5}$, $1:10^{-6}$ and tested on immunoblot strips of Dengue 2 virus antigens and control strips of uninfected C6/36 cells separated by SDS PAGE under non-denaturing conditions. Endpoint titers for the three rabbit sera of day 38 was calculated to be 1:10 000. The endpoint titers for the sera of day 66 were calculated to be 1×10^5 in both the immunoblot assay an the ELISA, respectively (data not shown).

Both, pre- and post-immunized serum were titrated at $1:10^{-2}$, $1:10^{-3}$, $1:10^{-4}$, $1:10^{-5}$, $1:10^{-6}$, $1:10^{-7}$ and tested in indirect IgG ELISA. The wells were coated with dengue 2 and uninfected C6/36 lysates at 1:250 dilution.

Table 3:

		$1:10^{-2}$	$1:10^{-3}$	$1:10^{-4}$	$1:10^{-5}$	$1:10^{-6}$	$1:10^{-7}$
Rabbit #1	Pre	-0.012	0.006	0.007	0.003	-0.002	0.001
	Post	0.623	0.127	0.02	0.004	0.001	-0.003
Rabbit #2	Pre	-0.012	0	-0.001	-0.003	0	-0.001
	Post	0.402	0.06	-0.007	0.008	-0.003	0.002
Rabbit #3	Pre	-0.008	0.03	-0.002	0.005	-0.002	-0.002
	Post	0.907	0.224	0.038	0.011	-0.001	-0.003

ELISA absorbance reading for pre- and post-immunized serum from each rabbit at different dilutions (Pre = pre-immune sera, Post = post immunization sera).

The titration results for post-immunized sera of each rabbit were plotted as shown Fig 4. The estimated endpoint titers for each rabbit post-immunized serum are 1:1000.

2.2 Testing of prebleed and post-immunization sera against dengue serotype 1, 3, and 4,Japanese encephalitis virus and West nile virus immunoblots

Each of the rabbit serum of day 38 was tested at 1:1000 dilution on immunoblot strips of Dengue 1, 2, 3, 4 and JE virus antigens plus control strips of uninfected C6/36 cells separated by SDS PAGE under non-denaturing conditions. It was shown that each rabbit post-immunization sera reacts with NS1 from dengue serotypes 1, 3 and 4 as well as with NS1 on Japanese encephalitis immunoblots.

Each of the rabbit serum of day 66 was tested at 1:1000 dilution on immunoblot strips of Dengue 1, 3, 4, WNV and JE virus antigens plus control strips of uninfected C6/36 cells separated by SDS PAGE under non-denaturing conditions. It was shown that each rabbit post-

immunization sera reacts with NS1 from dengue serotypes 1, 3 and 4 as well as with NS1 on Japanese encephalitis virus and West nile virus immunoblots.

To confirm the immunoblot assays Elisa cross reactivity assays were performed. The wells of microtiterplates were coated with DENV-1, DENV-3, DENV-4, JEV, WNV and uninfected cell lysates at 1:250 dilutions. The sera were sera from day 38 (Fig. 5A) and day 66 (Fig. 5B).

From the immunoblot assays as well as from the ELISA experiments it can be concluded that antibodies elicited by Dengue virus NS1 are cross reactive with all other Dengue serotypes, the JEV and the WNV.

2.3. **Conclusions**

- Rabbits immunized with mBN07 vaccine elicited antibodies that recognize authentic Dengue virus serotype 2 NS1.
- Very high immune response was observed where end points were $1:10^{-4}$ and $1:10^{-3}$ in both immunoblot assays and ELISA respectively.
- Antibodies elicited in the rabbits cross-reacted with all the other dengue serotypes (1, 3 & 4).
- The antibodies also cross-reacted with NS1 from a heterologous virus such as JEV and WNV.

3. Immunogenicity studies in mice

Female out-bred mice were immunized by the intraperitoneal route with mBN07 expressing Dengue virus NS1 in different amounts and schedules as shown below. mBN08, a MVA corresponding to mBN07 but not expressing NS1 and PBS served as control. The serum of the mice was used to check whether the antibodies generated in the mice were able to react on Western blots with NS1 proteins from the different flavivirus

serotypes and from the Japanese enzephalitis virus, respectively. The sera from the control mice were negative in all experiments.

The following groups were analyzed:

Group	No. of mice	1ST dose	Interval	2ND dose	Interval before bleeding
1	9	$1x10^7$ $TCID_{50}$ BN07 each (0.1ml of vaccine diluted 1:5 in PBS/mouse)	4 weeks	$1x10^7$ $TCID_{50}$ BN07 each (0.1ml of vaccine diluted 1:5 in PBS/mouse)	3 weeks
2	9	$1x10^7$ $TCID_{50}$ BN07 freeze-dried vaccine each (reconstituted in 1.2ml water per mouse)	4 weeks	$1x10^7$ $TCID_{50}$ BN07 freeze-dried vaccine each (reconstituted in 1.2ml water per mouse)	3 weeks
3	10	$1x10^7$ $TCID_{50}$ BN07 freeze-dried vaccine each (reconstituted in 1.2ml water per mouse)	3 weeks	$1x10^7$ $TCID_{50}$ BN07 freeze-dried vaccine each (reconstituted in 1.2ml water per mouse)	4 weeks
4	10	$1x10^7$ $TCID_{50}$ BN07 each (0.1ml of vaccine diluted 1:5 in PBS/mouse)	4 weeks	$1x10^7$ $TCID_{50}$ BN07 each (0.1ml of vaccine diluted 1:5 in PBS/mouse)	4 weeks
5	10	$1x10^7$ $TCID_{50}$ BN07 freeze-dried vaccine each (reconstituted in 1.2ml water per mouse)	4 weeks	$1x10^7$ $TCID_{50}$ BN07 freeze-dried vaccine each (reconstituted in 1.2ml water per mouse)	4 weeks

In the immunoblot experiments the following results were obtained:

GROUP	NO. OF MICE TESTED	DENV-1 POSITIVE (% POS)	DENV-2 POSITIVE (% POS)	DENV-3 POSITIVE (% POS)	DENV-4 POSITIVE (% POS)	JEV POSITIVE (% POS)
1	9	5 (55.5%)	9 (100%)	3 (33.3%)	7 (77.7%)	5 (55.5%)
2	9	8 (88.8%)	9 (100%)	8 (88.8%)	9 (100%)	5 (55.5%)
3	10	9 (90%)	10 (100%)	9 (90%)	10 (100%)	8 (80%)
4	10	8 (80%)	10 (100%)	9 (90%)	9 (90%)	4 (40%)
5	10	10 (100%)	10 (100%)	9 (100%)	10 (100%0	8 (80%)

Very similar experiments have been obtained with Balb/c mice: Mice were vaccinated with two shots of $1x10^8$ $TCID_{50}$ BN07 (freeze-dried and reconstituted with water) at days 0 and 21. The sera were obtained at day 42. 100% of the sera reacted with Dengue virus 2 NS1, 100 % of the sera reacted with NS1 proteins from all four Dengue virus serotypes and 75 of the sera reacted with the NS 1 protein of JEV. The results obtained with $1x10^8TCID_{50}$ non-freeze dried BN07 were as follows: 100% of the sera reacted with NS1 from all four Denguevirus serotypes. The sera recognized NS1 from JEV not as good as the sera obtained from mice vaccinated with freeze-dried BN07.

Conclusion:

- 100% of the mice immunized with BN07 had antibodies to DENV-2 NS1 with very strong response.
- The immune response of mice immunized with 1x10e7 TCID50 BN07 was as strong as the immune response of mice immunized with 1x10e8 TCID50 BN07 (data not shown)

- The best cross-reactivity percentage were observed at with mice immunized at 4 weeks interval and 4 weeks before bleeding .
- Mice immunized with the BN07 freeze-dried vaccine were observed to have much stronger response to NS1 compared to those immunized with the non-freeze dried vaccine.

References

Nimmannitya S, Kalayanaroo S, Nisalak A, and Innes B. 1990. Second attack of dengue hemorrhagic fever. Southeast Asian Journal of Tropical Medicine and Public Health, 21:699

Burke DS and Monath TP., 2001, Flaviviruses. In Fields Virology, Fourth Edition, Edited by David M Knipe and Peter M Howley. Published by Lippincott Williams and Wilkins, Philadelphia. Pages 1043-1125.

Flamand M, Megret F, Mathieu M, LePault, Rey FA and Deubel V., 1999. Dengue Virus Type 1 Nonstructural Glycoprotein NS1 is Secreted from mammalian cells as a soluble hexamer in a glycsylation-dependent fashion. J. Virol., 73:6104-6110.

Jang SK, Davies MV, Kaufman RJ and Wimmer E., 1989. Initiation of protein synthesis by internal entry of ribosomes into the 5' nontranslated region of encephalomycarditis virus RNA in vivo. J. Virol., 63:1651-60.

Lindenbach BD and Rice CM., 2001, Flaviviruses and their replication. In Fields Virology, Fourth Edition, Edited by David M Knipe and Peter M Howley. Published by Lippincott Williams and Wilkins, Philadelphia. Pages 991-1041.

Schlesinger JJ, Brandriss MW and Walsh EE., 1987. Protection of mice against dengue 2 virus encephalitis by immunization with the dengue 2 virus non-structural protein NS1. J. Gen. Virol., 68:853-7

Schesinger JJ, Foltzer M and Chapman S., 1993. The Fc portion of antibody to yellow fever virus NS1 is a determinant of protection against yellow fever encephalitis in mice. Virology. 192: 132-41

The techniques and procedures described in this description are familiar to a skilled practitioner(s) in the art of molecular biology and virology especially relating to Flavivirus virology and genetic manipulation of poxviruses. The techniques and procedures described can be found in detail in the following literature resources:

Molecular Cloning, A laboratory Manual. Second Edition. By J. Sambrook, E.F. Fritsch and T. Maniatis. Cold Spring Harbor Laboratory Press. 1989.

Virology Methods Manual. Edited by Brian WJ Mahy and Hillar O Kangro. Academic Press. 1996.

Molecular Virology: A Practical Approach. Edited by AJ Davison and RM Elliott. The Practical Approach Series. IRL Press at Oxford University Press. Oxford 1993. Chapter 9 : Expression of genes by vaccinia virus vectors.

Current Protocols in Molecular Biology. Publisher: John Wiley and Son Inc. 1998. Chapter 16, section IV: Expression of proteins in mammalian cells using vaccinia viral vector.

Antibodies, A Laboratory Manual. By Ed Harlow and David Lane. Cold Spring Harbor Laboratory Press. 1988.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 13, line 25.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution: ECACC
European Collection of Cell Cultures

Address of depositary institution *(including postal code and country)*:
Centre for Applied Microbiology & Research
Salisbury
Wiltshire SP4 OJG, United Kingdom

Date of deposit	Accession Number
August 30, 2000	00083008

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

In respect of all designated States to which such action is possible and to the extent that it is legally permissable under the law of the designated State, it is requested that a sample of the deposited microorganism be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g., EPC Rule 28 (4); UK Patent Rules 1995, Schedule 2, Paragraph 3; Australian Regulation 3.25(3); Danish Patents Act Sections 22 and 33(3) and generally similar provisions mutatis mutandis for any other designated State.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only

☒ This sheet was received with the international application

Authorized officer: O. Durand

For International Bureau use only

☐ This sheet was received by the International Bureau on:

Authorized officer

Form PCT/RO/134 (July 1992)

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 13, line 20.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution: ECACC
European Collection of Cell Cultures

Address of depositary institution *(including postal code and country)*:
Centre for Applied Microbiology & Research
Salisbury
Wiltshire SP4 OJG, United Kingdom

Date of deposit	Accession Number
December 7, 2000	00120707

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

In respect of all designated States to which such action is possible and to the extent that it is legally permissable under the law of the designated State, it is requested that a sample of the deposited microorganism be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation, e.g., EPC Rule 28 (4); UK Patent Rules 1995, Schedule 2, Paragraph 3; Australian Regulation 3.25(3); Danish Patents Act Sections 22 and 33(3) and generally similar provisions mutatis mutandis for any other designated State.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer: O. Durand	Authorized officer

Form PCT/RO/134 (July 1992)

31 JAN 2007

CLAIMS

1. Poxvirus vector harboring a DNA comprising an expression cassette comprising a transcriptional regulatory element and a sequence which codes for an entire Flavivirus NS1 protein or a part thereof that still elicits an immune response and optionally for a further peptide/protein, wherein the poxvirus is Modified Vaccinia Virus Ankara (MVA) strain BN deposited at the European Collection of Cell Cultures under number V00083008 or a derivative thereof and wherein the further peptide/protein is not an entire E-protein wherein the derivative of strain MVA-BN is characterized by having at least one of the following properties:

(i) capability of reproductive replication in chicken embryo fibroblasts (CEF) and in the baby hamster kidney cell line BHK, but no capability of reproductive replication in the human keratinocyte cell line HaCat,

(ii) failure to replicate in vivo,

(iii) induction of a higher immunogenicity compared to the known strain MVA 575 in a lethal challenge model and/or

(iv) induction of at least substantially the same level of immunity in vaccinia virus prime/ vaccinia virus boost regimes when compared to DNA-prime/ vaccinia virus boost regimes.

2. Poxvirus vector according to anyone of claims 1, wherein the Flavivirus is a mosquito-borne Flavivirus.

3. Poxvirus vector according to claim 2, wherein the mosquito-born Flavivirus is a Dengue virus.

4. Poxvirus vector according to claim 3, wherein the Dengue virus is Dengue virus serotype 2.

5. Poxvirus vector according to anyone of claims 1 to 4, wherein the sequence coding for the Flavivirus NS1 protein or part thereof is preceded by an ATG codon and a sequence encoding a glycosylation signal sequence and wherein the coding sequence is terminated by a stop codon of translation.

6. Poxvirus vector according to anyone of claims 1 to 5, wherein the transcriptional regulatory element is a poxvirus promoter.

7. Poxvirus vector according to anyone of claims 1 to 6, wherein the poxvirus vector is freeze-dried.

8. Pharmaceutical composition comprising a poxvirus vector according to anyone of claims 1 to 7 and a pharmaceutically acceptable carrier, diluent and/or additive.

9. Poxvirus vector according to anyone of claims 1 to 8 as a vaccine.

10. Poxvirus vector according to claim 9, wherein the vaccine is used to vaccinate against the Flavivirus or Flavivirus serotype from which the nucleic acid or the NS 1 protein or part thereof is derived and/or against one or more additional Flavivirus and/or Flavivirus serotype.

11. Use of a poxvirus vector according to anyone of claims 1 to 7 harboring a DNA comprising an expression cassette comprising a transcriptional regulatory element and a sequence which codes for an entire Flavivirus NS1 protein or a part thereof that still elicits an immune response and optionally for a further peptide/protein, wherein the further peptide/protein is not an entire E-protein for the preparation of a vaccine against the flavivirus or Flavivirus serotype from which the NS1 protein or

31 JAN 2007

part thereof is derived and/or against one or more additional Flavivirus and/or Flavivirus serotype.

12. Use of a poxvirus vector according to anyone of claims 1 to 7 harboring a DNA comprising an expression cassette comprising a transcriptional regulatory element and a sequence which codes for an entire Flavivirus NS1 protein or a part thereof that still elicits an immune response and optionally for a further peptide/protein, wherein the further peptide/protein is not an entire Eprotein and wherein the Flavivirus infection is an infection by the Flavivirus or Flavivirus serotype from which the nucleic acid, or the NS 1 protein or part thereof is derived and/or an infection by another Flavivirus and/or Flavivirus serotype in the manufacture of a medicament for the treatment or prevention of a Flavivirus infection.

13. Use of a poxvirus vector according to anyone of claims 1 to 7 harboring a DNA comprising an expression cassette comprising a transcriptional regulatory element and a sequence which codes for an entire Flavivirus NS1 protein or a part thereof that still elicits an immune response and optionally for a further peptide/protein, wherein the further peptide/protein is not an entire E-protein in the manufacture of a medicament for the vaccination against several flaviviruses or flavivirus serotypes.

14. Poxvirus vector according to claim 10 or, use according to anyone of claims 11 to 13, wherein the NS1 protein or - part thereof is derived from one Denguevirus serotype and wherein the vaccine protects an individual at least against the infection with at least two Denguevirus serotypes.

31 JAN 2007

15. Poxvirus vector or, use according to claim 14, wherein the vaccine protects an individual against the infection with all Denguevirus serotypes.

16. Poxvirus vector or, use according to anyone of claims 14 to 15, wherein the Denguevirus serotype and/or Flavivirus from which the NS1 protein is derived is Denguevirus serotype 2.

17. Poxvirus vector or, use according to anyone of claims 10 to 16, wherein the other/additional Flavivirus is selected from West Nile virus, Yellow fever virus and Japanese encephalitis virus.

18. Poxvirus vector or, use according to anyone of claims 1 to 7 and 9 to 17 or pharmaceutical composition according to claim 8, wherein the poxvirus vector or the pharmaceutical composition is administered in therapeutically effective amounts in a first inoculation ("priming inoculation") and in a second inoculation ("boosting inoculation").

19. Cell, preferably a human cell, comprising a poxvirus vector according to anyone of claims 1 to 7.

20. Kit for prime/boost immunization comprising a poxvirus vector according to anyone of, claims 1 to 7, 9 to 10 and 14 to 18 or a pharmaceutical composition according to claim 8 for a first inoculation ("priming inoculation") in a first vial/container and for a second inoculation ("boosting inoculation") in a second vial/container.