conditions, such as virus passage in vivo, would occur. Moreover, VEEV TC-83 is capable of replicating in mosquito cells, and infecting mosquitoes following vaccination (32); therefore, its transmission by mosquitoes remain possible.

Ideally, live arbovirus vaccine strains should not be transmissible by arthropod vectors, because circulation among reservoir hosts could lead to unforeseen changes that might include increased virulence. This is especially true for attenuated strains, produced from wild-type viruses that rely on small numbers of attenuating mutations that may be subject to reversion, or for genetically modified strains that might evolve in unanticipated ways if they underwent vector-borne circulation. The former risk was underscored by the detection of the VEEV TC-83 vaccine strain in mosquitoes collected in Louisiana during 1971 (32), an area outside the epizootic/epidemic that was restricted to Texas.

The development of infectious cDNA for alphaviruses opened an opportunity to explore their attenuation by extensively modifying the viral genomes, an approach that might minimize or exclude the reversion to the wt, pathogenic phenotype. Moreover, the genomes of such alphaviruses can be engineered to contain RNA elements that would be functional only in cells of vertebrate, but not insect, origin. Thus, such extensive mutations could prevent transmission of the genetically modified viruses by mosquito vectors.

Despite its importance as an emerging human and animal pathogen, its potential as a biological weapon and concerns about application of attenuated alphaviruses, the prior art is deficient in methods of generating attenuated strains of alphaviruses that are capable of replicating only in vertebrate cells. The present invention fulfills this long-standing need and desire in the art.

SUMMARY ~~OF THE INVENTION~~

In one aspect, there is provided a method of generating attenuated, recombinant alphavirus incapable of infecting mosquitoes, comprising: cloning an encephalomyelocarditis virus (EMCV) internal ribosomal entry site (IRES) wherein the IRES is capable of expression in mammalian cells but not insect cells; and a capsid gene downstream of envelope glycoprotein genes with the capsid gene at the 3' end of the subgenomic region just upstream of the 3' UTR, wherein the capsid is expressed in an IRES-dependent manner and the envelope protein genes are expressed in a cap-dependent manner.

In one aspect, there is provided a method of generating an attenuated, recombinant alphavirus incapable of infecting mosquitoes, comprising: (i) inactivating the subgenomic promoter of an alphavirus; and (ii) cloning an encephalomyelocarditis virus (EMCV) internal ribosomal entry site (IRES) between the end of nonstructural protein 4 (nsP4) coding sequence and initiating AUG of a subgenomic RNA coding sequence of the alphavirus wherein the IRES is capable of expression in mammalian cells but not insect cells; and generating the live, attenuated, recombinant alphavirus.

In one embodiment of the present ~~invention~~disclosure, there is provided a method of generating an attenuated recombinant alphavirus. Such a method comprises cloning the internal ribosomal entry site of encephalomyelocarditis virus (EMCV IRES) between the end of non-structural protein 4 (nsP4) coding sequence and initiating AUG of a subgenomic RNA coding sequence of an alphavirus instead of natural 5' UTR of subgenomic RNA. In a further related embodiment of the present ~~invention~~disclosure, there is an attenuated recombinant alphavirus generated by the method discussed supra.

In one aspect, there is provided an attenuated, recombinant alphavirus, comprising: an alphavirus nucleic acid having (i) an inactivated subgenomic promoter, and (ii) an insertion of an encephalomyelocarditis virus (EMCV) internal ribosomal entry site (IRES) between the end of nonstructural protein 4 (nsP4) coding sequence and initiating AUG of a subgenomic RNA coding sequence of the recombinant alphavirus, wherein the IRES is capable of expression in mammalian cells but not insect cells; and wherein the alphavirus is attenuated.

In one aspect, there is provided an attenuated, recombinant alphavirus comprising:

a) a capsid gene positioned at the 3' end of the subgenomic region, downstream from envelope glycoprotein genes and upstream from the 3' UTR of the subgenomic RNA of the alphavirus; and

b) an encephalomyelocarditis virus (EMCV) internal ribosomal entry site (IRES) introduced between the end of the envelope glycoprotein genes and the positioned capsid gene, wherein the IRES is capable of expression in mammalian cells but not insect cells; and

wherein the capsid is expressed in an IRES-dependent manner and the envelope protein genes are expressed in a cap-dependent manner; and wherein the alphavirus is attenuated.

In one aspect, there is provided a vector comprising a nucleotide sequence encoding the attenuated, recombinant alphavirus as defined herein.

In one aspect, there is provided a host cell comprising and expressing the vector as defined herein.

In yet another related embodiment of the present ~~invention~~disclosure, there is provided a vector comprising nucleotide sequence encoding the attenuated recombinant alphavirus and a host cell comprising and expressing this vector.

In one aspect, there is provided a pharmaceutical composition comprising: the attenuated, recombinant alphavirus as defined herein and a pharmaceutically acceptable carrier.

In a yet another related

embodiment of the present ~~invention~~disclosure, there is provided a pharmaceutical composition. This composition comprises the attenuated recombinant alphavirus discussed supra and a pharmaceutically acceptable carrier. In one aspect, there is provided an immunogenic composition comprising: the attenuated, recombinant alphavirus as defined herein. In a related embodiment of the present ~~invention~~disclosure, there is provided an immunogenic composition. This immunogenic composition comprises the attenuated recombinant alphavirus described herein.

In one aspect, there is provided the pharmaceutical composition as defined herein for use in protecting a subject from infections resulting from exposure to an alphavirus.

In another related embodiment of the present ~~invention~~disclosure, there is provided a method of protecting a subject from infections resulting from exposure to an alphavirus. Such a method comprises administering an immunologically effective amount of the immunogenic composition comprising the attenuated, recombinant alphavirus described herein, thereby protecting the individual from the infections resulting from the exposure to the alphavirus.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1D show replication of the recombinant, EMCV IRES-encoding, VEEV TC-83-derived viruses in BHK-21 cells. **Figure 1A** is a schematic representation of the designed viral genomes, infectivities of the in vitro-synthesized RNAs in the infectious center assay, virus titers at 24 h post transfection of 1 mg of the in vitro-synthesized RNAs into BHK-21 cells, and sizes of the plaques, formed by indicated viruses in BHK-21 cells at 48 h post transfection. Arrows indicate functional subgenomic promoters. Filled boxes indicate EMCV IRES. **Figure 1B** shows alignment of the subgenomic promoter-containing fragment of the VEEV TC-83 genome and the corresponding fragment of the VEEV/mutSG/IRES. The position of the promoter is indicated by open box. The start of the subgenomic RNA in the VEEV TC-83 genome and the beginning of the EMCV IRES are indicated by arrows. The mutations, introduced into the VEEV/mutSG/IRES genome are shown in lower case letters. **Figure 1C** shows plaques, formed in BHK-21 cells by viruses, harvested at 24 h post transfection. **Figure 1D** shows replication of the viruses after transfection of 1 mg of the in vitro-synthesized RNAs into BHK-21 cells.

Figures 2A-2C show mutations found in the plaque-purified VEEV/mutSG/IRES variants, which demonstrated more efficient replication in BHK-21 cells, and the effect of the defined adaptive mutations on VEEV TC-83 and VEEV/mutSG/IRES replication. **Figure 2A** shows the list of the mutations, found in the genomes of plaque isolates, compared to the published sequence of VEEV TC-83 (24). **Figure 2B** is a schematic representation of the VEEV TC-83 and VEEV/mutSG/IRES genomes, having either one or both identified mutations, and the infectivity of the in vitro-synthesized viral RNAs in the infectious center assay. Functional subgenomic promoters are indicated by arrows, and EMCV IRES by filled boxes. **Figure 2C** shows replication