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To the Minister for the Environment and Public Transport
Ministery of Infrastructure and Water Management
Mr Ch.A. Jansen
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DATE 30 April 2025
REFERENCE CGM/250430-01
SUBJECT Advice on tightening conditions on inserts in alphavirus replicons

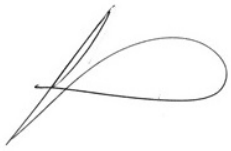
Dear Mr Jansen,

In response to new scientific knowledge on the formation of infectious virus-like vesicles (VLVs), COGEM is tightening up its earlier generic advice on downscaling containment requirements for work with alphavirus and flavivirus replicons. COGEM therefore notifies you of the following.

Summary:
In 2022 COGEM advised that laboratory work with viral replicons derived from alphaviruses and flaviviruses can be performed on a lower containment level when certain conditions are met. In viral replicons, certain structural genes are removed so that virus particles can no longer be formed and, in theory, cannot spread. New genes can be inserted at the positions of the deleted genes. One of the conditions for generic downscaling is that the new genes introduced into the replicon do not restore the removed functions. As the insertion of specific genes from other viruses in alphavirus replicons can lead to the formation of transmissible virus-like vesicles (VLVs), the use of structural genes from three virus families was ruled out.
In view of the growing number of scientific publications that describe the formation of VLVs when structural genes from viruses in families other than those previously excluded are used, COGEM advises to further tighten up the conditions for the use of such inserts. Instead of excluding structural genes from specific virus families, the Commission advises that alphavirus replicons containing genes or combinations of genes that give the replicon the capacity to spread, should be excluded from generic downscaling to a lower containment level. COGEM is of the opinion that by including this condition in its previous generic advice on assigning containment levels for work with alphavirus replicons, human and environmental safety will be safeguarded.

The attached report contains COGEM's advice and a discussion of the underlying reasoning.

Yours sincerely,



Professor Sybe Schaap
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c.c.

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Advice on tightening conditions for generic downscaling of containment requirements for the use of transgenes in alphavirus replicons

COGEM Advisory Report CGM/250430-01

1. Introduction

In 2022 COGEM issued a generic advice on downscaling contained use work with 'naked' replicons and viral replicon particles (VRPs) derived from viruses from the genera *Alphavirus* and *Orthoflavivirus*.[1] In that report COGEM advised that downscaling containment requirements for work with alphavirus and flavivirus replicons is possible as long as a number of conditions are met, including the condition that no transgenes may be used that can complement the removed functions. Specifically for alphavirus replicons, this means that sequences that code for the envelope proteins of viruses from the families *Togaviridae*, *Rhabdoviridae* and *Retroviridae* are excluded from generic downscaling because they can lead to the formation of infectious virus-like vesicles (VLVs – spherules on the plasma membrane where replication complexes may be assembled[2]), enabling further spread of the replicon.

A growing number of scientific publications describe the formation of infectious VLVs when a number of different transgenes are used in alphavirus replicons. These infectious VLVs can also be formed when the transgenes used are obtained from viruses in families other than those mentioned above. They also report that the infectious titre of the VLVs can be further increased by passaging the VLVs in cells, and that it is possible for the replicon RNA to spread from cell to cell via infectious VLVs. However, there are no data available on the possibility of spread between animals or humans.

On the basis of the latest scientific knowledge, COGEM is of the opinion that, as a precautionary measure, the scope of the generic advice on the use of transgenes in alphavirus replicons on a lower containment level should be further restricted.

2. Alphaviruses: replication and assemblage of virus particles

Alphaviruses (genus *Alphavirus*, the only genus in the family *Togaviridae*) have a wide host range and are capable of infecting birds, mammals, fish and insects.[3,4] They can spread via bloodsucking insects, especially mosquitoes.[3] The genus *Alphavirus* has 32 species,[4] including various pathogenic species in humans and/or animals, such as *Alphavirus chikungunya* (Chikunga virus, CHIKV), *Alphavirus rossriver* (Ross River virus, RRV), *Alphavirus semliki* (Semliki Forest virus, SFV), *Alphavirus sindbis* (Sindbis virus, SINV) and *Alphavirus venezuelan* (Venezuelan equine encephalitis virus, VEEV).[5]

Alphavirus particles have a diameter of about 65–70 nm and contain a positive single-stranded RNA genome of 9.7 to 11.8 kb.[4] Replication of the virus genome takes place in the cytoplasm of the host cell. The genome contains two large open reading frames (ORFs). The non-structural proteins (nsP1–nsP4) are translated from the ORF at the 5'-end of the genome as a polyprotein; the individual proteins are formed following cleaving by the viral protease in nsP2. These proteins are involved in the replication of the viral RNA and form the replication complex.[5,6] The ORF at the 3'-end of the genome encodes the

structural proteins. This ORF is expressed from a subgenomic mRNA, which is transcribed from an internal promoter, 26S, situated in the area between the structural and non-structural ORF.[3,6] Viral and cellular proteases cleave the structural polyproteins in the individual proteins (C, E3, E2, 6K and E1). In some cases, (-1) frame shifting during the translation leads to the production of an extra protein called TF.[7] The non-coding regions are at both ends of the genome and the viral RNA has a 5'-cap structure and a poly(A) tail at the 3'-end.

The RNA in the virus particle is enclosed by capsid proteins (C) and together they form the nucleocapsid. The protein coat is surrounded by a lipid membrane containing the glycoproteins (envelope proteins, E1 and E2) involved in binding to and infection of the host cell.[8] The glycoproteins therefore have a direct influence on the host range.[4] The assemblage and budding of new virus particles take place on the plasma membrane of the host cell.[9]

3. Alphavirus replicons: naked replicons and replicon particles

Viral replicons are RNA or DNA molecules that can replicate independently and are derived from RNA or DNA viruses. This advice only concerns RNA replicons derived from viruses from the genus *Alphavirus*. These replicons have various applications, including in research on viral genome replication, as vaccines and as possible cancer immunotherapies or gene therapies.[10,11,12,13,14,15,16,17] In the production of replicons, a distinction is made between 'naked' viral replicons and viral replicon particles (VRPs).

3.1 Naked viral replicons

In naked replicons derived from alphaviruses, the ORF encoding the structural genes is deleted. The non-structural genes and RNA sequences necessary for genome replication, such as the non-coding regions, are still present, which means that the replicon RNA is capable of independent replication. Because the structural genes have been deleted, new virus particles cannot be produced after infection. A transgene can be inserted at the position of the structural ORF and expressed from the subgenomic 26S promoter. In some replicons, a second 26S promoter is introduced into the replicon genome so that multiple (trans)genes can be expressed. Introducing viral 2A peptide sequences or viral internal ribosome entry sites (IRES) elements can promote the expression of multiple transgenes. SFV, SINV and VEEV, in particular, are used in the construction of replicon systems.[18,19]

Naked viral replicons can be delivered into the cell as an RNA molecule by electroporation or transfection. Naked RNA can also be packaged into non-viral or synthetic particles, such as nanoparticles (lipid nanoparticles, LNPs), which can be taken up by the cell by means of endocytosis.[19] Another way of administration is to produce an RNA replicon from a DNA plasmid delivered into the cell, called DNA-launched RNA replication (DREP). The plasmid is transported to the nucleus, where the replicon RNA is transcribed, for example by a CMV promoter on the plasmid. Translation and replication of the replicon RNA takes place in the cytoplasm following the release of the RNA molecule from the cell nucleus. If the replicon also contains a selection gene, such as the gene that encodes neomycin resistance, stable replicon cell lines can be selected in which the replicon RNA remains in the cells.

Examples of such naked alphavirus replicons are the self-amplifying mRNA (samRNA) vaccines, which have been much studied in recent years. Clinical trials are being held into the use of several self-replicating replicons, mostly derived from VEEV, as vaccines or cancer therapies.[15,16,17,19,20] Recently, approval was given for the first samRNA vaccine to be authorised for use in the EU, Kostaive.[21,22] This vaccine against COVID-19 is derived from the vaccine strain VEEV-TC-83 and is formulated as LNPs. The advantage of using samRNA vaccines over standard mRNA vaccines is that a lower dose can be used, because the mRNA itself can replicate in the host cell.

3.2 Viral replicon particles

Viral replicon particles (VRPs) consist of the replicon RNA enclosed in viral structural proteins. For the production of the VRPs, the missing structural genes are provided *in trans*. VRPs are capable of infecting cells, but the lack of structural genes in the replicon RNA means that no new VRPs can be produced. As infection occurs just once, further spread is prevented.

Alphavirus VRPs are mainly produced by co-transfection of the replicon RNA with one or more helper constructs, from which the structural proteins are translated. The helper constructs are often also derived from alphaviruses from which the coding sequence for the non-structural proteins has been removed. A distinction is made between single helper strategies, in which the structural genes are provided on a single helper RNA, and split-helper systems. In the latter production systems, the C gene and the genes encoding the glycoproteins are situated on two different helper RNAs.[23,24] As far as COGEM has been able to ascertain, production systems in which the structural genes are provided from plasmids have not been described. Stable cell lines that produce the structural proteins have been described, but are little used.[25]

VRPs are also being studied for potential use in vaccines or for oncolytic applications.[16]

4. Formation of infection virus-like vesicles

Naked replicons and replicon particles are considered to be biologically contained, because the structural genes of the alphavirus are removed, making further spread to uninfected cells impossible. If the alphavirus envelope proteins (glycoproteins E1 and E2) in alphavirus replicons remain intact and the deletion in the structural genes is therefore limited to the C gene, virus-like versicles (VLVs) may be formed. These are spherules on the plasma membrane of the infected cell where replication complexes may be assembled.[2] The result is that the replicon RNA is surrounded by a membrane to which the envelope proteins are anchored. As the capsid proteins are absent, these VLVs are unstable and have a different appearance than the virus particle or VRP. The formation of VLVs is less efficient than the production of virus particles. However, such VLVs are infectious and to a certain degree capable of infecting other cells.[26,27]

Infectious VLVs[a] can also be formed when transgenes that code for viral envelope proteins from other virus families are introduced into alphavirus replicons, for example during the development of vaccines against various infectious diseases. In the literature there are now reports of infectious VLVs being formed from alphavirus replicons with envelope proteins of rhabdoviruses,[2,28,29,30,31,32] retroviruses,[33,34] filoviruses[35] and coronaviruses.[36] VLVs can be serially passaged in cell lines, which can result in the acquisition of mutations in the non-structural genes leading to more efficient production and increased titres of VLVs.[2,30]

VLVs are not only seen as an unwanted by-product in the production of replicons; research is also being done on the use of infectious VLVs as vaccines against various infectious diseases[28,30,31,32,35,37] and as a potential immunotherapy against cancer.[38] These applications require a high titre, which can be achieved, as mentioned above, by serial passaging of the infectious VLVs in cell cultures.[2,29,30,36] Other alterations can be made to the replicon to increase the titre of infectious VLVs, and multiple transgenes can be introduced to increase the efficiency of the production of VLVs. For example, Zhang et al. (2024) described the production of a VLV vaccine against Ebola in which an alphavirus replicon based on VEEV was used.[35] The alphavirus structural proteins were replaced by the glycoprotein (GP) of the Ebolavirus leading to limited formation of VLVs. However, when the VP40 matrix protein of the Ebolavirus was also introduced, the infectious VLVs could be produced more efficiently. The titre could be significantly increased by serially passaging the GP/VP40 VLVs in cells.[35]

In addition, research is being conducted on inserting immunomodulating transgenes into replicons with the aim of attenuating the interferon response to the replicon. Replication of the replicon RNA initiates a stronger immune response (i.e. the production of cytokines, including interferon) than when non-replicating mRNA is used. A too strong immune response causes degradation of the RNA, decreasing transgene expression. Immunomodulating transgenes in the replicon, often of viral origin, inhibit the interferon response.[39,40] In addition, modifications can also be made to the mRNA nucleotides, such as the use of (N1-methyl)pseudouridine or 5-(hydroxy)methylcytosine instead of unmodified uracil or cytosine to avoid recognition by the immune system.[19] The application of immunomodulating transgenes in VLVs derived from SFV-VSV-G replicons has recently been studied, including for potential oncolytic application.[37]

5. Previous COGEM advice

In 2022 COGEM issued a generic advice on the requirements for working with naked replicons and VRPs derived from viruses from the genera *Alphavirus* and *Orthoflavivirus*, on a lower containment level.[1] COGEM was of the opinion that a generic downscaling of containment requirements for these replicons was possible under certain conditions.

The conditions were drawn up to limit the risk of the replicon spreading or the formation of replication-competent virus. First, to prevent the restoration of removed functions by complementation or

a. Different terms are used in the scientific literature for infectious VLVs. For example, they are also called 'pseudoviruses', 'infectious microvesicles', 'self-propagating membrane-enveloped vesicles', 'self-propagating hybrid replicon particle' (PRP) and 'live attenuated vaccines'.

recombination, the cells used should not contain related viruses. Other conditions prescribe a minimum set of deletions of structural genes from the replicon and require that the alphavirus VRPs are produced with a split-helper system in which the C and envelope encoding sequences are strictly separated. Also, the transgenes used must not be capable of restoring or complementing the removed functions. Given the possibility of infectious VLVs being formed when alphavirus replicons are used, the excluded transgenes also included the sequences that code for the envelope proteins of viruses from the families *Togaviridae*, *Rhabdoviridae* and *Retroviridae*.

6. Considerations

Naked replicons and replicon particles are considered to be biologically contained, because the alphavirus structural genes are removed, rendering them incapable of spreading to uninfected cells. However, a growing number of scientific publications describe the generation of infectious VLVs from replicons derived from alphaviruses. The transgenes used in these replicons are coding sequences from envelope proteins of viruses from various families. Infectious VLVs generated (intentionally or unintentionally) during work with replicons derived from alphaviruses can lead to further spread of the replicon RNA from cell to cell. Because the C protein is missing, VLVs are produced less efficiently than the original virus particles and it is assumed that the VLVs are less stable. However, several publications report that the infectious titre of VLVs can be increased by adaptation during passaging in cell cultures and that combinations of different transgenes can also lead to more efficient formation of VLVs. Whether infectious VLVs can be shed by animals or humans is not known; also, as far as COGEM is aware, there are no data on the spread of infectious VLVs between animals and/or humans.

Furthermore, insertion of immunomodulating sequences into alphavirus replicons, often of viral origin, is known to increase the efficiency of replication of the replicon because the interferon response is inhibited. While this leads to the improved expression of transgenes, it may also lead to more efficient spreading of VLVs. However, there are no data available to support this.

In the light of developments described above and the uncertainties surrounding the shedding and spread of infectious VLVs, COGEM is of the opinion that, as a precautionary measure, generic downgrading of containment requirements for work with replicons should not be permitted when the formation of infectious VLVs is a possibility.

In its previous advice on work with replicons, COGEM excluded the use of envelope proteins from certain virus families from the generic environmental risk assessment as a requirement for working on a lower containment level with alphavirus replicons.[1] However, this list has proved to be non-limiting in the light of the recent publication of several studies describing the production of VLVs when other viral envelope proteins were used. The formation of infectious VLVs has now been described for replicons that express envelope proteins of rhabdoviruses,[2,28,29,30,31] retroviruses,[33,34] filoviruses[35] and coronaviruses.[36] However, COGEM does not rule out the possibility that other proteins with fusogenic characteristics (i.e. proteins that can facilitate the fusion of cell membranes) are also capable of making VLVs infectious. Combinations of genes may also lead to more efficient production of infectious VLVs

(as described for the VLV vaccine against Ebolavirus), with the accompanying increased probability of shedding and spread.

7. Advice

COGEM previously stated the following general conditions for downscaling the containment requirements for work with alphavirus replicons:[1]

- the cells used must not contain any related alphaviruses;
- the transgenes used must not complement the removed functions (this includes sequences that code for the envelope proteins of viruses from the families *Togaviridae, Rhabdoviridae* and *Retroviridae*).

Because envelope proteins from other virus families may also lead to (unintentional) formation of VLVs, and the risks of infectious VLVs being formed have not yet been fully identified, COGEM advises that the condition in its previous advice excluding sequences that code for the envelope proteins of viruses from the families *Togaviridae*, *Rhabdoviridae* and *Retroviridae* should be replaced by a new condition:

- work with alphavirus replicons containing transgenes or combinations of transgenes that give the replicon the capacity to spread should be excluded from generic downscaling of containment requirements.

Examples of transgenes that can facilitate the spread of replicons are viral structural proteins with fusogenic characteristics, including those of endogenic retroviruses (ERVs[b]) and combinations of potential fusogenic and immunomodulating proteins.

COGEM is of the opinion that by including this broader condition in its previous generic advice on assigning containment levels for work with alphavirus replicons,[1] human and environmental safety will be safeguarded.

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