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REFERENCE CGM/180316-01

SUBJECT Advice on containment levels for replication-deficient adenoviral vector systems

Dear Mrs Van Veldhoven,

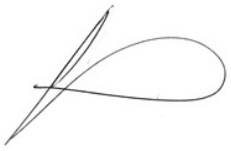
A study has been carried out for COGEM on the environmental risks of contained use activities involving vectors based on biologically contained adenoviruses and adeno-associated virus. The research report gives the Commission reason to submit the following advice on adenovirus-based vectors.

Summary:
Vector systems based on adenoviruses are frequently used in biomedical research. In recent years increasingly advanced vector systems have been developed based on these viruses and scientific understanding of and experience with the use of these systems is increasing. To streamline the authorisation procedure for these systems, COGEM commissioned a study to determine whether or not activities involving these vector systems are eligible for a generic downgrading of containment level.

Based on the resulting report, COGEM distinguishes between three adenoviral vector systems. For each of these systems, COGEM recommends appropriate containment levels for the production of the vectors and activities involving them. Regarding activities, COGEM makes a further distinction between laboratory experiments and experiments on test animals.

The grounds on which COGEM has reached its conclusions and the resulting advice are set out in the enclosed report.

Yours sincerely,



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Generic advice on containment levels for replication-deficient adenoviral vector systems

COGEM advice CGM/180316-01

1. Introduction

The appropriate containment level in the laboratory, animal houses, etc. for an activity involving genetically modified organisms (GMOs) is determined primarily by the pathogenicity class of the parental organism. The containment level of an activity may be downgraded if, for example, the GMO is biologically contained or has a reduced pathogenicity. In response to questions from licensing authorities, COGEM regularly advises that activities involving biologically contained viruses[a] may be carried out at a lower safety level without compromising human and environmental safety.

In biomedical research, vector systems based on adenoviruses (AdV) and adeno-associated viruses (AAV) are frequently used. In recent years advanced AdV- and AAV-based vector systems have been developed and scientific understanding of and experience with the use of these systems is increasing. To streamline the authorisation procedure for these systems, COGEM commissioned a literature study to determine whether or not activities involving these vector systems may be considered for a generic downgrading of containment level. The study was carried out by Ameco Adviesgroep Milieubeleid in cooperation with the Erasmus University Medical Center and the Netherlands Institute for Neuroscience.

Based on the information in the resulting report, 'Downgrading of containment levels for activities involving replication-defective AAV and adenoviral vectors',[1] and on supplementary information from the literature, COGEM concludes that generic downgrading of containment levels for activities involving AdV- and AAV-based vector systems is possible. In the present advice, COGEM explains this position for adenoviral (AdV) replication-deficient vector systems. A separate advice on AAVs will be published later.

2. Adenovirus

Adenoviruses belong to the *Adenoviridae* family and are found in vertebrate animals such as humans, simians, rodents, cattle, snakes, pigs, fish and birds. The family *Adenoviridae* consists of five genera (*Atadenovirus, Aviadenovirus, Ichtadenovirus, Mastadenovirus* and *Siadenovirus*) containing a total of 74 species.[2,3] New adenoviruses are regularly discovered. The genus *Mastadenovirus* is the largest and contains 45 species.[2]

[a] Viruses may be biologically contained because the viral genome is unable to replicate (replication deficient) or because the virus particle cannot be assembled, for example due to the absence of capsid proteins (replication incompetent). In both cases any infection is restricted to the first round of infection.

The adenoviruses are categorised on the basis of their nucleotide sequence similarities, hemagglutination profile and sensitivity to neutralising antisera.[4] These serotypes are indicated by a number. Some species contain serotypes with different host tropisms. For example, *Human mastadenovirus C* contains human adenoviruses (e.g. Human adenovirus type 2 and 5: HAdV-2, HAdV-5), Bovine adenovirus type 9 (BAdV-9) and Simian adenovirus type 13 (SAdV-13)).[2]

2.1 Pathogenicity characteristics of adenoviruses

Most adenovirus species have a narrow host range restricted to a single or just a few very closely related animal species. The virus infects the respiratory system, the gastrointestinal tract and the eyes, and sometimes the urinary tract and liver.[2,4,5] Infections are generally subclinical or present with mild symptoms, without any need for medical treatment, and are usually self-limiting. In patients with a much weakened immune system, the viruses can cause severe infections of the kidneys and lungs, with possibly fatal consequences.[4] Following an infection, adenoviruses may episomally persist as inert DNA in the cell or latently in tissues.[1,5] Adenovirus is transmitted by direct or indirect contact with infected persons, via faeces and urine, and by aerogenic transmission.[4,6] Some adenovirus infections can be treated with antiviral agents, but in many cases these drugs are ineffective.[2,5,7]

2.2 Pathogenicity classification of adenoviruses by assessment agencies worldwide

COGEM has assigned all the adenoviruses on which it has so far issued advice to pathogenicity class 2.[8] The Belgian Biosafety and Biotechnology Unit (SBB) and the Swiss Federal Office for the Environment (FOEN) have assigned adenoviruses to risk class 2.[9,10] The German Central Committee on Biological Safety (ZKBS) has assigned adenoviruses of horses, sheep, pigs, ducks, geese, chickens, budgerigars and fish to the lowest risk group (group 1)[11] and the other adenoviruses to risk group 2. In their assessments, COGEM, FOEN, WIV-ISP and ZKBS take pathogenicity to humans, animals and plants into consideration.

As far as is known by COGEM, adenoviruses have never been assigned to a higher risk group as 2 by any authority worldwide. COGEM has no reason to assume that there are any adenoviruses that should be assigned to a higher risk group.

The classifications by foreign assessing agencies are used as reference and background information on the considerations of COGEM.

2.3 Structure and genome organisation of adenoviruses

Adenovirus particles consist of a linear double-stranded DNA molecule enclosed by a protein capsid.[2,12] The protein capsid consists of hexons, pentonbases and various smaller proteins.[1,5] Attached to the pentonbases are ‘fibres’, which protrude above the surface of the capsid and bind to a receptor on the host cell.[4] The hexons, pentonbases and fibres contain antigenic determinants and are important in the recognition of the virus by the immune system.[5] The hemagglutination and neutralisation profiles of adenovirus are determined primarily by these capsids.

The adenovirus genome varies in size per species and contains between 26 and 49 kilobasepairs (kbp). The genome is made up of a 'packaging signal' ψ (psi), an 'early' (E) and 'late' (L) region, and the 'inverted terminal repeats' (ITRs).[4]

The ITRs are located at either end of the genome and function as the 'origin of replication'. The E region is expressed shortly after the virus enters the cell and consists of various transcription units, each of which encodes several proteins: E1A, E1B, E2A, E2B, E3 and E4.[1,3,4,5] The E1A proteins have a role in inducing replication of the virus and expressing the other E genes, and in cell cycle regulation. The E1B proteins block the programmed cell death (apoptosis) of the infected cell. In addition, the 'minor capsid' protein pIX is expressed at the 3' end of the E region. This protein is part of the capsid and is involved in transduction and tropism.[13] The E2A and E2B proteins are necessary for replication of the viral genome: E2A encodes the single-stranded (ss)DNA binding protein; E2B encodes the DNA polymerase and the terminal precursor protein. The E3 proteins block the inflammatory response to the virus, among other things by inhibiting the natural killer cells (NK cells), cytotoxic T-lymphocytes and tumor necrosis factor (TNF). The E4 region encodes proteins involved in cell cycle regulation and in the expression of the L region.[3,4,5] The L region contains genes which encode structural proteins involved in the assembly of the virus particle.[3,4] The packaging signal ψ is involved in packaging the viral genome into the virus particle.[3] Replication and assembly of the virus take place in the cell nucleus of the host cell.[5]

2.4 Adenoviral vectors

Biomedical research on adenoviruses includes the development of viral vectors, for example for use in the development of vaccines or in gene therapies.[5] This involves removing certain sections of the adenoviral genome or inserting transgenes at the site of a deletion for the purpose of expressing specific proteins. Also, vectors are being created from different serotypes of one or more adenovirus species (hybrid vectors), for example by exchanging parts of hexons or fibres from an adenovirus into the vector backbone (hexon/fiber swaps).[14,15]

3. Adenoviral replication-deficient vector production systems

AdV replication-deficient vector production systems consist of a combination of an adenovirus from which one or more transcription units within the E region have been removed, and a production cell line that *in trans* complements these deletions and the missing viral traits they encode for. The deletions in the virus are such that the produced vector is replication deficient (no longer able to replicate its genome). There are three AdV replication-deficient production systems: type 1, type 2 and helper dependent (HD) production systems.[1,5] The systems are described briefly below. They are illustrated in Alba *et al*. (2005)[16] and in the research report by Ameco (Figure 7, page 35).[1]

3.1 Type 1 AdV production systems

The first-generation replication-deficient AdV vectors have a deletion in the E1 region (ΔE1-AdV). An expression cassette containing the transgene of interest can be inserted here. The E1 region is involved, among other things, in inducing viral replication and expressing the other E genes.

Various production cell lines have been developed for producing first-generation vector particles. These cell lines differ from the sequences of the inserted E1 region, the promoter and the poly-A signal. In some production systems the E1A and E1B genes are inserted into various locations in the genome of the cell line. The most commonly used production cell lines are HEK293, 911 and PER.C6.[1] Kovesdi *et al*. (2010) give an overview of E1 complementary production cell lines. [17] Some type 1 production systems have been altered to prevent homologous recombination by ensuring there is no sequence homology in the E1 sequences of both the vector particle and the production cell line.[1,17] This minimises the possibility of the creation of a replication-competent adenovirus (RCA) during production, which in turn minimises the possibility of 'contamination' of the vector batch. The production systems based on the PER.C6 cell lines, among others, possess these alterations.

It is also possible to remove the E3 region or part of the E3 region (ΔE3) to increase the cloning capacity of a first-generation AdV vector. The function of the E3 region (blocking the inflammatory response to the virus) does not have to be complemented by the production line cells.[1,3,5]

3.2 Type 2 AdV production systems

In second generation replication-deficient AdV vectors, besides a deletion in the E1 region (and possibly in the E3 region) there are also deletions in the E2A, E2B and/or E4 regions (ΔE2A, ΔE2B, ΔE4), which means there is no replication of the viral genome and no expression of the late genes,[1,5] thus increasing the transgene cloning capacity.

Production cell lines of the second type do not only complement the E1 functions, but also the E2A, E2B and E4 functions. These cell lines are derived from type 1 production cell lines. An overview of these is given in the research report prepared by Ameco *et al*. (2017) and in Kovesdi *et al*. (2010) (in Table 3 and Table 2 respectively).[1,17]

3.3 HD (helper dependent) AdV production systems

HD AdV vectors are also called high capacity, gutted or gutless vectors. They contain only the ITRs and the packaging signal ψ of the adenovirus.[1,5] The remainder of the genome consists of the expression cassettes with the transgene or transgenes of interest. HD vectors therefore have a high cloning capacity. Non-coding 'stuffer' DNA may also be added to the genome to ensure its length falls between a certain minimum and maximum so that it will fit inside the capsid.[1,18,19]

In HD production systems the functions of the adenoviral genes must be complemented by the production cell lines to enable the generation of vector particles.[1,16,20] However, as constitutive expression of several adenoviral gene products leads to cytotoxic effects,[1,16,21] the adenoviral genes in HD production systems are expressed via co-infection with a complementing helper vector. A ΔE1-AdV vector may be used as a helper vector in combination with a production cell line that expresses the E1 region of the adenovirus (type 1 AdV production system).

To prevent the ΔE1-AdV helper genome being packaged in the vector particle and thus becoming a 'contaminant' in the vector batch (helper vector particles) alongside the intended vector, HD production systems are adapted by inserting site-specific recombination systems. The Cre-*loxP* system is usually used for this; *loxP* sites are introduced into the ΔE1-AdV helper vector. These sites are the binding sites for the Cre recombinase. In a production cell line expressing the Cre recombinase, the packaging signal (ψ) of the helper vector is removed by recombination of the *loxP* sites after the ΔE1-AdV helper vector has infected the production cell, thus preventing the helper vector particle from being packaged.[1,20,22] To further reduce the possibility of 'contamination' with the packaged ΔE1-AdV helper genome, the HD vector can be optimised by removing the overlapping sequences on either side of ψ in the HD vector to prevent recombination between the HD vector and the Δψ-ΔE1-AdV helper vector.[1,23]

4. Considerations

This advice concerns replication-deficient AdV production systems and vectors in which the introduced transgene does not encode a harmful gene product. AdV vectors with a donor sequence encoding a harmful gene product are not included, because in advance carrying out a generic risk assessment of such recombinants, would be extremely difficult according to COGEM.

Replication-deficient AdV vectors are biologically contained because they lack transcription units within the E1 region, which means they cannot replicate their genome. Once a cell is infected, the effects will remain limited to the first round of infection. As a result of the deficiency, the vectors are unable to replicate autonomously in a cell, unable to cause a productive infection, and unable to spread in the environment. The environmental risk of producing and using replication-deficient AdV vectors is therefore determined by the possibility of the unintentional generation of RCA, and the spread of RCA in the environment.

Given that the production of a vector batch involves other risks than the application of a vector, COGEM elaborates these activities separately below.

4.1 Production of replication-deficient AdV vectors

Replication-deficient AdV vectors lack one or more transcription units from the E1 region (ΔE1-AdV) and, depending on the production system, also multiple transcription units from other E regions (ΔE2A, ΔE2B, ΔE3, ΔE4, HD). Vector particles are produced and replicated in specially developed cell lines that complement the missing trait or traits. Various studies have shown that homologous recombination can occur during the production of replication-deficient AdV vector particles if the flanking sequences of the E1 region in the viral vector overlap with the sequence of the E1 region in the production cell line. In some production systems this can lead to RCA.[1,16,24]

4.1.1 Possibility of the generation of RCA in type 1 AdV production systems

If use is made of a type 1 production system (production of ΔE1-AdV or ΔE1ΔE3-AdV vectors), RCA may be formed after recombination of the E1 region. If the ΔE1 vector is used, the RCA will be more or less the same as a wild type adenovirus. If the ΔE1ΔE3 vector is used, the RCA will be attenuated because it lacks the E3 region (att-RCA).[1,24] If a transgene is introduced into the E3 region, the att-

RCA will contain the transgene (transgene att-RCA). COGEM points out that although the vector particles of ΔE1ΔE3 vectors are replication competent, the attenuation will prevent a productive infection *in vivo* because the particles will be cleared by the immune system. As far as is known by COGEM, ΔE3 adenoviruses have never been found in nature, which supports the claim that ΔE1ΔE3 vectors cannot survive in the environment.

If use is made of vector backbones containing elements from various adenoviruses (for example, hybrid vectors, see 2.4), any RCA formed will be a hybrid virus particle. As all adenoviruses belong to pathogenicity class 2, COGEM has no reason to assume that this will be different for hybrid adenoviruses. Therefore, COGEM is of the opinion that the pathogenicity of the hybrid RCA will be comparable with that of the parent organisms.

In some production systems there is no homology between the E regions of the vector and the cell line, which means no homologous recombination can take place and the possibility of RCA being generated is negligible. Gao *et al*. (2000) and Schnieder *et al*. (2000) have confirmed this empirically for such systems.[1,25,26] The possibility of RCA being generated is also negligible in production systems that make use of the PER.C6 cell line in combination with specific vectors with no sequence homology in the sequences of both E1 regions.[1,27] Therefore, COGEM is of the opinion that the possibility of RCA being formed is negligibly small if the sequence data confirm that the production cell line and replication-deficient AdV vector used do not show sequence homology in the E1 region.

4.1.2 Possibility of the generation of RCA in type 2 AdV production systems

In second generation vectors, besides the E1 region, the E2A, E2B and/or E4 regions are also removed. However, the transcription units of E1, E2A and E2B are needed for the replication of the vector genome. COGEM considers the possibility of RCA being formed during production by second generation vectors to be negligible, because this requires multiple recombinations. If recombination occurs, new replication-deficient vectors will be produced in which just one of the regions mentioned is reintroduced.

4.1.3 Possibility of the generation of RCA in HD AdV production systems

If use is made of an HD production system, the AdV will not be able to generate RCA via homologous recombination, because the vector only contains the ITRs and the packaging signal. The possibility of RCA being formed from the helper vector cannot be ruled out however, it can be reduced by the presence of a site-specific recombination system (for example, the Cre-*loxP* system). Palmer *et al*. (2003) have shown that 10^{13} vector particles produced using an HD production system are 0.01% to 0.02% contaminated with helper vectors.[1,22] Analogous to the type 1 production systems, these helper vector particles can generate RCA via homologous recombination.

To summarise, COGEM comes to the following conclusions:

- During the production of replication-deficient AdV vectors using type 1 and HD production systems, there is a possibility that RCA will be generated through homologous recombination. The

Commission is therefore of the opinion that this type of production should be carried out at containment level ML-II.
- When a type 1 or HD production system involving a helper vector/cell line combination without sequence homology in the E region is used, or when a type 2 production system is used, the Commission considers the possibility of RCA being generated to be negligible. The Commission is therefore of the opinion that the environmental risk will be negligible if the production takes place at containment level ML-I.

4.2 Activities involving replication-deficient AdV vectors

For experiments involving adenoviral vector batches to become eligible for containment level I, three aspects need to be taken into consideration. First, there must be no RCA present in the vector batch (see 4.1). Second, the possibility of RCA generation must be negligible. Third, COGEM considers unintentional exposure of humans and animals to high concentrations of replication-deficient adenoviral vector particles to be undesirable. Although these particles are biologically contained and unable to disperse in the environment, and therefore present no environmental risk, they are infectious and may infect laboratory staff or, during *in vivo* experiments, animals not involved in the experiment. In these cases, the occupational, safety, health and welfare (ARBO) regulations should apply to ensure the safety of laboratory staff. As far as is known by COGEM, there is no comparable set of regulations for animals. In view of this, the Commission considers that for the safety of animals not directly involved in the experiment, the GMO legislation is the most appropriate and is of the opinion that for *in vivo* experiments to become eligible for downgrading to containment category D-I (Animal Housing), there must be no release of high concentrations of replication-deficient adenoviral vector particles.

Regarding the first consideration, COGEM notes that RCA may be present in vector batches produced using type 1 and HD production systems and in which the E1 regions of the viral vector and production cell line show similarities because of overlapping sequences. The Commission is therefore of the opinion that these batches must be tested for the absence of any RCA before activities involving these vectors can be eligible for downgrading of containment level. An adequate bioassay of quantitative PCR (qPCR) could be used for this purpose. A qPCR will identify the number of genome copies, but will not provide a measure of the amount of infectious virus; the value found by the test will be an overestimate of the real RCA titre. COGEM is of the opinion that an adequate PCR will be sufficient if the detection limit is <1 RCA genome copies per 5×10^7 replication-deficient infectious vector particles.[28] If use is made of type 1 or HD production systems without sequence homology in the E1 region, or if use is made of type 2 production systems, the Commission is of the opinion that vector batches have not to be tested on the presence of RCAs beforehand.

With regard to the other two considerations – no RCA generation during experiments and the unwanted exposure of laboratory animals not involved in the experiment to vector particles – it is important to identify when they could happen. COGEM draws a distinction between *in vitro* and *in vivo* experiments.

4.2.1 Possibility of RCA generation during in vitro experiments

During transduction experiments the effects on cells will be limited to the first round of infection because the vector particle is replication-deficient. The vector genome will persist in the cell for a short time as inert DNA in episomal form.[33] In theory, if cells are contaminated with adenovirus (for example, when using primary mammalian cells), co-infection with a vector particle after complementation by homologous recombination could lead to the generation of RCA. COGEM notes that contamination of cells with adenovirus will soon be noticed because of the evident occurrence of virus-induced cell death. In addition, latent or persistent adenovirus infections in cultures of permissive host cells are rare and, as far as is known by COGEM, it has not been demonstrated that latent infected cell lines can generate RCA after infection with replication-deficient vectors.

Also, it could be possible during the course of an experiment for cells to become contaminated with wild type adenovirus. COGEM considers that this form of contamination is highly unlikely – even at containment level ML-I – because experimental activities on cells and tissues are carried out in a safety cabinet to prevent microbial contamination of cell lines. Besides, safe microbiological techniques are used in these work spaces as a matter of routine. The Commission therefore considers the possibility of cells being contaminated with wild type adenovirus from outside to be negligible. Moreover, COGEM is of the opinion that any RCA generated during the experiment will not present a higher risk than wild type viruses.

Taking the above into consideration, COGEM is of the opinion that:

- the possibility of a vector batch initially free of RCA generating RCA during *in vitro* treatments is negligible;
- replication-deficient AdV vector batches that are free of RCA may continue to be used at containment level ML-I;
- a vector batch is free of RCA when it is produced using a type 2 production system or using a type 1 or HD production system without sequence homology in the E1 regions;
- if use is made of another production system, the vector batch must first be tested by means of a sensitive and validated RCA assay, and that the assay will be sufficient if the detection limit is <1 RCA genome copies per 5 x 107 replication-deficient infectious vector particles.

4.2.2 Possibility of RCA generation during in vivo experiments

Vector functionality is investigated not only in laboratory experiments, but also in experiments on test animals. For these experiments to be eligible for containment level DM-I, the vector batch must be RCA free. In addition, the possibility of the generation of RCA in the animals must be negligible.

If a laboratory animal is permissive or semi-permissive for an administered vector, but this vector is not based on an adenovirus for which humans are permissive, COGEM considers the possibility of the vector causing a productive infection in humans to be negligible. As far as is known, only human and simian adenoviruses can cause productive infections in humans. Should a laboratory animal be

permissive or semi-permissive for the administered vector, and this vector can lead to productive infections in humans (for example, a replication-deficient vector based on HAdV is given to non-human primates, cotton rats or Syrian hamster),[29,30] the vector particle can generate RCA if the animal is infected with an adenovirus, the same cell is infected with the vector and the virus, and a homologous recombination between the genome of the vector particle and the adenovirus occurs after complementation. The possibility of a co-infection increases if high doses are injected locally into a tissue or organ. COGEM therefore considers that for these *in vivo* experiments to be eligible for containment level DM-I it is essential that the laboratory animals must not show any signs of acute adenovirus infection before the experiment.

COGEM also points out that it considers the probability of mice, rats, hamsters, guinea pigs and rabbits carrying adenovirus to be negligible, because laboratory animal centres adhere to the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA).[31] This body states that laboratory animals must be free of certain infectious agents. To ensure this, mice, rats, hamsters, guinea pigs and rabbits in animal laboratory centres are regularly screened for adenovirus.[32]

If a laboratory animal is non-permissive for the vector to be used in the experiment, even if it is an adenovirus carrier, the sequence homology with the vector will be very limited indeed. This will be the case, for example, when a mouse, rabbit, dog, pig or sheep is administered with a replication-deficient vector based on HAdV. COGEM therefore considers that the possibility of homologous recombination in such *in vivo* application will be negligible, and that in this case the risks to human health and the environment will be negligible if the experiment is carried out at containment level DM-I.

To summarise, COGEM is of the opinion that if a laboratory animal is non-permissive for the vector to be administered, the risks to human health and the environment will be negligible if the experiment is carried out at containment level DM-I. If a laboratory animal is permissive or semi-permissive for a vector that is to be administered, and this vector can cause a productive infection in humans, COGEM is of the opinion that the laboratory animal must not show any signs of an acute adenovirus infection before the experiment. If these conditions are met, the COGEM considers that the risks to human health and the environment will be negligible if the experiment is carried out at containment level DM-I.

4.2.3 Possibility of replication-deficient vector particles being released during in vivo experiments

COGEM considers it inadvisable for *in vivo* experiments to be downgraded to D-I level if the animal excretes replication-deficient AdV vector particles. Although these particles do not pose any environmental risk, they are infectious and could, for example, be transmitted to other animals via aerosols or via direct or indirect contact. Based on the findings in the research report and as far as is known by COGEM, laboratory animals no longer excrete any infectious replication-deficient AdV vector particles a week after the vector is administered.[1] The Commission is therefore of the opinion that it is acceptable to transfer laboratory animals to a D-I containment facility one week after the

experiment, and considers it unnecessary to carry out a quantitative PCR to confirm the absence of vector genomes in a body fluid.

5. Advice

Based on the considerations discussed above, COGEM comes to the following advice:

- The Commission advises that the production of first-generation and HD replication-deficient AdV vectors should be carried out at containment level ML-II, because RCA can be generated by means of homologous recombination. If it can be demonstrated by a sensitive and validated method that no RCA is present in the vector batch (detection limit < 1 RCA genome copies per 5 x 10^7 replication-deficient infectious vector particles), COGEM considers that the risks to human health and the environment will be negligible when *in vitro* and *in vivo* activities involving these batches are carried out at containment level I.

- COGEM advises that the production of second generation vectors or first-generation and HD replication-deficient AdV vectors without sequence homology in the E1 regions of the vector and production cell line (determined from sequence data) can be carried out at containment level ML-I. The Commission considers that the possibility of RCA being generated through homologous recombination in these systems is negligible. The Commission also considers that the risks to human health and the environment of *in vitro* and *in vivo* activities involving these vector batches are negligible when they are carried out at containment level I. The Commission is of the opinion that the batches do not need to be tested for the absence of RCA before the experiment.

- If a laboratory animal is non-permissive for a replication-deficient AdV vector that is to be administered, the Commission considers that the risks to human health and the environment will be negligible if the experiment is carried out at containment level DM-I. If a laboratory animal is permissive or semi-permissive for a replication-deficient AdV that is to be administered, the Commission considers it essential that the animal does not show any signs of an acute adenovirus infection before the experiment in order to prevent the generation of RCA through homologous recombination after complementation. If these conditions are met, the COGEM considers that the risks to human health and the environment will be negligible if the experiment is carried out at containment level DM-I.

- COGEM considers that for *in vivo* experiments to become eligible for downgrading to containment level D-I, it is essential that the animals are held in a DM-I facility for at least a week before the experiment takes place. The Commission considers that after this week, the possibility that the animals will still excrete vector particles is negligible, and is of the opinion that human and animal safety will be guaranteed if the animals are housed at containment level D-I.

6. Observations on occupational safety, health and welfare

COGEM observes that during the production of replication-deficient AdV vector particles, large quantities of infectious particles are generated which, although they present no risk to the environment, are capable of infecting laboratory staff or other workers. If a worker is unintentionally exposed to vector particles, this may have adverse consequences. After infection the particles persist for a short time in the cell as inert DNA in episomal form and adenoviral genes may be expressed at a low level.[33] As a consequence, the transduced cells may induce an immune reaction, with possible harmful effects on the host. This is an occupational health and safety aspect and safety measures need to be taken to prevent any health effects from occurring.

In addition, COGEM observes that, should a worker have a clinical or subclinical adenovirus infection and during the experimental activities be accidentally infected with a replication-deficient first-generation vector or helper vector, the generation of RCA as a consequence of complementation and homologous recombination with the wild type adenovirus cannot, in theory, be entirely ruled out. *In vitro* complementation of ΔE1 vectors with wild type adenovirus, both between different serotypes within the same adenovirus species (intraspecies) and between different adenovirus species (interspecies), has been described in the literature.[34] Intraspecies recombination also occurs naturally in adenoviruses, in which hexons, pentonbases and fibre fragments (shaft/knob region) may be exchanged.[35,36,37,38] Interspecies recombination also occurs in adenoviruses.[38,39] Although a hybrid or other RCA could be generated during homologous recombination, COGEM is of the opinion that the RCA thus generated will not be more pathogenic than the wild type virus with which the worker was already infected, and does not consider that such incidents will present an environmental risk.

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