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DATE	24 March 2021
REFERENCE	CGM/210324-02
SUBJECT	Advice on generic environmental risk assessment of clinical studies with replication-deficient AdV vectors

Dear Ms Van Nieuwenhuizen,

Further to a request for advice on a broad licence application for vaccine trials with replication-deficient adenoviral (AdV) vectors, COGEM has drawn up a generic environmental risk assessment for clinical applications with replication-deficient AdV vectors for the purpose of streamlining the licensing procedures for these studies.

Summary:
Vectors derived from adenoviruses (adenoviral (AdV) vectors) are the most commonly used vectors in clinical gene therapy trials. A distinction is made between vectors that can replicate (replication-competent) and vectors that cannot replicate (replication-deficient).

On a number of occasions in the past COGEM has issued advice on clinical studies in which AdV vectors were used. Further to a request for advice on a broad licence application for clinical vaccine trials with replication-deficient AdV vectors, COGEM has prepared a generic environmental risk assessment for clinical applications that use these vectors. This generic environmental risk assessment is limited to replication-deficient AdV vectors in which at least the E1 region of the viral genome has been removed.

Replication-deficient AdV vectors are attenuated and biologically contained, and because they are replication-deficient they cannot spread in the environment. COGEM is of the opinion that, given the properties of replication-deficient adenoviral (AdV) vectors, the risks to human health and the environment of clinical trials with these vectors are negligible, provided a number of conditions are met. This generic environmental risk assessment can simplify and streamline the authorisation process for clinical trials, because it can provide the basis for drawing up a set of standard licence conditions (*vergunning onder vaste voorwaarden* – VoV) for these applications.

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

Yours sincerely,

Professor Sybe Schaap
Chair of COGEM

c.c.
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Generic environmental risk assessment of replication-deficient adenoviral vectors in clinical trials

COGEM Advisory Report CGM/210324-02

1. Introduction

The most widely used vectors in clinical gene therapy trials are viral vectors derived from adenoviruses (AdV vectors). More than 500 clinical trials with AdV vectors have been carried out worldwide.[1] The advantages of these vectors over other vectors include their ability to achieve high transduction efficiency and gene expression (although temporarily), their broad cellular tropism and the fact that these vectors can transduce both dividing and non-dividing cells.[2]

1.1 Generic environmental risk assessment

In 2018 COGEM issued a generic advice on the appropriate containment level for activities involving replication-deficient AdV vector systems under contained use.[3] Replication-deficient AdV vectors are biologically contained. On several occasions in recent years COGEM has issued advice on clinical studies involving the use of replication-deficient AdV vectors. This present generic environmental risk assessment was prepared in response to a broad application for the use of a replication-deficient AdV vector derived from human adenovirus type 26 (HAdV-26) in clinical vaccine trials, either on their own or in combination with a modified vaccinia virus Ankara (MVA) vector. This environmental risk assessment describes the conditions under which clinical trials with replication-deficient AdV vectors based on adenoviruses of human origin or from non-human primates may be carried out. It can form the basis for drawing up a simplified authorisation procedure with a set of standard licence conditions, (*vergunning onder vaste voorwaarden* – VoV) for clinical trials with replication-deficient AdV vectors. The properties of the vectors and other factors and information relevant to the environmental risk assessment are discussed further below.

2. General properties of adenoviruses

2.1 Taxonomy and types of adenoviruses

Adenoviruses belong to the *Adenoviridae* family and are found in vertebrates such as mammals, reptiles, amphibians, fish and birds. The family *Adenoviridae* consists of five genera (*Atadenovirus*, *Aviadenovirus, Ichtadenovirus, Mastadenovirus* and *Siadenovirus*) and 80 species.[4] Human adenoviruses (HAdV) and adenoviruses of other mammals (including bats, cattle, dogs, deer, dolphins, horses, mice, sheep and non-human primates) belong to the genus *Mastadenovirus*. This genus contains 50 species, including 7 species of HAdV called *Human mastadenovirus A* to *G* and 9 adenovirus species from non-human primates (SAdV) called *Simian mastadenovirus A* to *I*.[4] New adenoviruses are discovered regularly. Defining a new adenovirus strain used to be based on serology; nowadays, however, new types are mainly identified using genomic sequence analysis. The criteria for typing human adenoviruses are constantly evolving and there is no consensus on this among scientists.[5] Within species, adenoviruses are further subdivided into serotypes or types, which are indicated by a number.

For example, the species *Human mastadenovirus A* includes HAdV types 12 and 18. At the moment more than 100 different types of HAdV have been identified.[5]

2.2 Structure and genome organisation of adenoviruses

The genome of adenoviruses consists of a linear double-stranded DNA molecule. The size of the genome varies between 26 and 49 kilobase pairs (kbp) per species and it is surrounded by an icosahedric protein coat (capsid).[6,7,8] The particles are about 70 to 90 mm in diameter. The capsid of the virus particle consists of three major capsid proteins – protein II (hexon), III (penton base) and IV (fibre) – and several minor capsid proteins, such as IIIa, VI and VIII. The virus particle of many mastadenoviruses also contains the structural protein IX.[7,8] The fibres are anchored in the penton bases and protrude above the surface of the capsid. They consist of a tail, a shaft and a knob, which binds to the receptor on the host cell.[6,7,8] The hexons, penton bases and fibres contain antigenic determinants and are important in the recognition of the virus by the immune system.[9] Several receptors have been identified for HAdV. The coxsackie and adenovirus receptor (CAR) is used by almost all HAdV species, with the exception of a number of HAdV-B types that infect cells via CD46.[8,9] Several other receptors and co-receptors are also known for HAdV.[8,9]

The core of the virus particle consists of the DNA genome, with the covalently linked terminal protein (TP) at both 5' ends, which forms a complex with proteins V (only present in mastadenoviruses), VII, X (also called Mu or μ), IVa2 and the viral protease.[6,7] The double-stranded DNA genome can be subdivided into several regions: the packaging signal ψ (psi), the early (Early or E) and late (Late or L) regions, and the inverted terminal repeats (ITRs) at both ends of the genome. The ITRs function as the origin of replication, where DNA replication of the genome begins. The TP proteins act as primers for replication.[6,7]

The E region is expressed shortly after the virus enters the cell and consists of various transcription units, each of which codes for several proteins: E1A, E1B, E2A, E2B, E3 and E4.[6,10] The E1A proteins play a part in inducing replication of the virus and expressing the other E genes, and are involved in cell cycle regulation. The E1B proteins block the programmed cell death (apoptosis) of the infected cell.[6,9,10] The presence of the E1 region in the viral genome is essential for replication of the viral genome. Mastadenoviruses also express the minor capsid protein pIX at the 3' end of the E1 region. This protein is part of the capsid of the virus particle and is instrumental in stabilising the virus particle (it helps to make the capsid more robust) and regulating transcription, and is involved in the reorganisation of proteins in the cell nucleus.[11]

The E2A and E2B proteins are necessary for replication of the viral genome: E2A codes for the single-stranded DNA binding protein (DBP) and E2B for the DNA polymerase and the precursor of the TP protein (pTP).[6,10] The E3 proteins inhibit the host's antiviral response following infection and block the inflammatory response to the virus, including by inhibiting the functioning of natural killer cells (NK cells), cytotoxic T lymphocytes and tumour necrosis factor (TNF). As far as is known, adenoviruses with a deletion in the E3 region have never been found under natural conditions, so it is assumed that

viruses with this deletion cannot survive in the environment. The E4 region codes for proteins involved in cell cycle regulation and in the expression of the L region.[6,9] The L region contains genes which code for the structural proteins needed to assemble the virus particle.[6,9] The packaging signal ψ is involved in packaging the viral genome into the virus particle.[6]

Much of the adenoviral infection cycle takes place in the host cell nucleus, including expression of the E region, viral DNA replication and expression of the late viral genes, as well as assembly and maturation of the new virus particle. The new virus particles are released after lysis of the cell.[6,9]

2.3 Pathogenicity and transmission of adenoviruses

Adenoviruses can spread by direct contact, via coughing and sneezing, or via contaminated objects or surfaces. Some adenoviruses can also spread by the oral/faecal route. Adenoviruses can also spread by water (for example in swimming pools), but this is less common.[12]

In general, HAdV infect the upper and lower respiratory tracts, but conjunctivitis and infections of the gastrointestinal tract are also common. Infections of the bladder and liver are less common.[9] More than 80% of the diagnosed infections are observed in children under four years old.[13] Infections with HAdV are usually self-limiting and lead to mild symptoms, although cases of serious illness, sometimes with a fatal outcome, have been reported.[14] In immunocompromised individuals, infection with HAdV is associated with increased morbidity and mortality.[9,14] Sometimes HAdV can be persistent, with low-level shedding of the virus over a long period.[15] No specific therapy for adenoviral infections is available.

Adenoviruses are generally considered pathogenic only to their specific host.[9] Adenoviral infections in animals show a similar range of symptoms as in human adenoviral infections. For example, adenoviruses in non-human primates (SAdV) are generally not associated with serious illness in these animals.[16,17] As far as is known, adenoviruses from non-human primates do not circulate in the human population. Some cases of zoonotic (from animal to human) and zooanthroponic (from human to animal) transmission have been described, but no serious symptoms were observed.[18]

3. AdV vectors in clinical trials

AdV vectors of human origin are the most commonly used vectors in clinical gene therapy trials.[1] The vectors are used to deliver a repaired version of a gene to treat genetic diseases, as a vaccine to induce an immune response against pathogens or as a treatment against cancer. For cancer treatment, replicating AdV vectors, or oncolytic vectors, are usually used and are designed to destroy cancer cells by means of a lytic infection cycle.[19] Replication-competent AdV vectors are not covered by this generic advice, which is limited to a generic environmental risk assessment for replication-deficient AdV vectors.

As the efficacy of AdV vectors can be hampered by immunity against a number of common human adenoviral viruses, such as HAdV-5, there is an ongoing search for ‘rare’ adenoviruses that can serve as a backbone for AdV vectors. Examples include vectors based on HAdV-26 and HAdV-35[20,33] and vectors derived from adenoviruses isolated from non-human primates.[21]

In replication-deficient AdV vectors at least the E1 region of the viral genome has been deleted, rendering the vectors biologically contained and unable to replicate independently. Depending on the type of vector, in addition to the E1 deletion (ΔE1) several transcription units within the E region are also deleted (ΔE2A, ΔE2B, ΔE3, ΔE4), or just both ITRs and the packaging signal remain present. For the production of these vectors, a production cell line is used that complements the missing viral properties required for the production of vector particles in trans.

There are three types of replication-deficient AdV vectors:[3,9,10]

- First-generation replication-deficient AdV vectors. These vectors have a deletion of the essential E1 region (ΔE1-AdV); in addition, all or part of the E3 region may be deleted (ΔE1ΔE3-AdV). The production of first-generation AdV vectors takes place in a complementary cell line in which the E1 genes are expressed. The function of the E3 region (blocking the immune response to the virus) does not have to be complemented by the production line cells, because the E3 genes do not code for proteins needed for the production of the vector.[6,9,10] In many cases the transgene is inserted at the position of the E1 region, but it can also be inserted in the E3 region of the genome.

  Various production cell lines have been developed for production of the first-generation vector particles. The differences between these cell lines lie in the sequences of the inserted E1 region and the regulatory signals (the promoter and the poly A signal) used to express the E1 region. In some production systems the E1A and E1B genes are inserted at different locations in the genome of the cell line. The most commonly used production cell lines are HEK293, 911 and PER.C6.[10, 22]

- Second-generation replication-deficient AdV vectors. In addition to a deletion of the E1 region (and possibly the E3 region), these vectors also have deletions in the E2A, E2B and/or the E4 regions (ΔE2A, ΔE2B, ΔE4). This prevents replication of the viral genome and expression of the late genes,[9,10] and increases the capacity for cloning transgenes. Production cell lines of second-generation AdV vectors complement the E2A, E2B and E4 functions in addition to the E1 function. These cell lines are often derived from the production cell lines used for first-generation AdV vectors.[22]

- Helper dependent (HD) vectors: These vectors contain only the ITRs and the packaging signal ψ (psi) of the adenovirus.[9,10,26,27] Some vectors also contain a small non-coding sequence from the 'right-hand' part of the AdV genome.[23] The rest of the genome of the HD vectors consists of the expression cassettes with the transgene or transgenes of interest. 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 virus coat.[10,23,24,25] These vectors are also called 'high capacity', 'gutless' or 'gutted' vectors.

  A production cell line for HD AdV vectors must complement the functions of all essential adenoviral genes to enable the generation of vector particles.[10,23] Since the constitutive expression of a number of adenoviral gene products in a cell leads to cytotoxic effects, HD

vectors are produced in the presence of a complementary helper vector.[10,19,23,26,27] In most cases a ΔE1ΔE3 AdV vector acts as a helper vector, with production taking place in a cell line that expresses the E1 proteins of AdV.[23] The presence of E3 in the helper vector, however, leads to increased production.[28] To prevent contamination with the helper vector during production, a site-specific recombination system, such as the Cre-*loxP* system, is often used. In this system, the helper vector contains *loxP* sequences on both sides of the packaging signal. After expression of Cre recombinase by the helper cells or from the helper vector genome, the packaging signal is deleted from the helper vector genome to prevent it from being packaged in a virus particle.[26,29,30] Although the amount of helper vector contamination can be greatly reduced – to less than 0.1-0.4% – by using Cre-*loxP* and similar systems,[31] a further purification step, for example by density gradient centrifugation, is always required to further reduce the amount of helper vector.[23]

ΔE1 and ΔE3 AdV vectors can lead to 'leaky' expression of the remaining early and late adenoviral genes, which can induce a strong immune response to the infected cells.[19] These first-generation AdV vectors are therefore often used as vaccines with the aim of inducing an immune response in the vaccinated individual to give protection against infectious diseases.[a] First-generation AdV vectors are also used as anti-tumour therapies, for example by expressing the tumour antigen p53 in cancer cells.[b] Although there have been gene therapy clinical trials with these AdV vectors,[19] ΔE1 AdV vectors are not considered to be optimal for gene therapy. The above-mentioned 'leaky' expression, which leads to the possible clearing of transduced cells, blocks long-term transgene expression.[19] HD AdV vectors from which all AdV genes have been removed are able to persist episomally in the cells for a longer period.[23]

3.1 Hybrid vectors

In some cases replication-deficient vectors are constructed so that one or more genes can be exchanged between different types or species of AdV. These 'hybrid' vectors are designed, for example, to optimise production of the relevant replication-deficient AdV vectors in existing production cells, which usually express the E1 proteins from HAdV type 5. For example, exchanging the E4 ORF6 sequence in ΔE1 AdV vectors derived from HAdV-26 or HAdV-35 with the corresponding sequence derived from HAdV-5 optimises the production of these vectors in PER.C6 cells (in which the E1 region of HAdV5 is present).[22,32,33] Also, parts of hexon fibres from another adenovirus ('hexon/fibre swabs') can be introduced into the vector backbone by exchange, with the aim of modifying the tropism of the AdV vector or circumventing the existing immunity against the vector.[34,35,36] The tissue tropism of AdV vectors can also be modified by inserting a peptide containing arginine-glycine-aspartate (RGD) into the fibre knob.[37,38]

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[a] At the moment three vaccines based on replication-deficient AdV vectors have been authorised for use. Zabdeno (Johnson & Johnson) is used in combination with a vaccine based on a modified vaccinia virus Ankara (MVA) to prevent Ebola. Like the vaccine against SARS-CoV-2 from the same company, Zabdeno is derived from HAdV type 26 from which E1 and a large part of E3 have been removed. AstraZeneca's SARS-CoV-2 vaccine is also a first-generation AdV vector and is derived from the chimpanzee adenovirus Y25 (ChAdY25).

[b] An example of this is Gendicine, a replication-deficient ΔE1 and ΔE3-AdV vector with a functional p53 gene as transgene. It has been approved in 2003 for commercial use in China by the State Food and Drug Administration.

4. Previous COGEM advice

COGEM has assigned all the adenoviruses on which it has so far issued advice to pathogenicity class 2.[39] In 2018 it issued a generic advice on the appropriate containment level for work with replication-deficient adenoviral vector systems under contained use (production and in vitro and in vivo activities).[3] COGEM assigned laboratory work with vector systems in the absence of replication-competent adenovirus to containment level ML-I. In addition, COGEM has on several occasions advised on clinical trials with replication-deficient adenoviruses.[40,41,42,43]

Under an accelerated assessment procedure, the European Medicines Agency (EMA) has approved the AdV-based Ebola vaccine Ad26.ZEBOV-GP for placing on the European market.[44] In 2019, COGEM issued a confidential[c] positive advice on this marketing authorisation.[45] In 2020 COGEM advised on a vaccination study with Ad26COVS1, which fell under the emergency regulation with a shortened authorisation procedure for gene therapies against COVID-19.[46] In early 2021, COGEM issued two confidential advisory reports in which it concluded that the environmental risks of authorising two vaccines against SARS-CoV-2, one from AstraZeneca and one from Janssen-Cilag, are negligible.[47,48]

5. Formation of replication-competent adenovirus during the production of replication-deficient AdV vectors

Replication-deficient adenoviral vectors are produced using production cell lines that express the adenoviral proteins necessary for the replication. Depending on the combination of the vector design and the production cell line used, there is a chance that replication-competent adenovirus (RCA) will be formed. RCA formation associated with the different types of vector is discussed in more detail below.

5.1 Chance of RCA formation during the production of first-generation AdV vectors

If use is made of a first-generation 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 virtually the same as a wild-type adenovirus. If the ΔE1ΔE3 vector is used, the RCA will be strongly attenuated because it lacks the E3 region.[10,49] If a transgene is introduced into the E3 region, the RCA will contain the transgene. If use is made of vector backbones composed of different adenoviruses, RCA formation will lead to the creation of a hybrid virus particle.

In some production systems there is no sequence overlap between the E1 regions of the vector and the cell line, which means no homologous recombination can take place and the probability of RCA formation is negligible. This is the case for most ΔE1-AdV vectors when they are produced in PER.C6 cell lines, in which the E1 genes are under the control of the human phosphoglycerate kinase (PGK) promoter.[10,50] Gao et al. (2000) and Schnieder et al. (2000) have confirmed this empirically for similar systems.[10,51,52] A point to consider is that no sequence overlap between the AdV vector and the production cell line may be present in order to completely rule out the possibility of recombination. For

[c] This concerns advice on an application for marketing authorisation of a medical application in the EU. Such cases and licence applications are always declared confidential by the European Medicines Agency (EMA). Under EMA guidelines, COGEM may not publish its advice or make it publicly available in any way.

example, recombination has been reported during the production of a first-generation ΔE1 AdV vector in PER.C6 cells, which was brought about because a sequence of 177 nucleotides at the 3' end of E1B from the vector matched the sequence in PER.C6 cells.[53]

5.2 Chance of RCA formation during the production of second-generation AdV vectors

In second-generation AdV vectors the E2A, E2B and/or E4 regions are deleted in addition to the E1 region. However, the transcription units of E1, E2A, E2B and E4 are needed for the replication of the vector genome. For second-generation AdV vectors, therefore, multiple recombinations would be needed during production to form RCA. The chance of multiple recombinations occurring is negligible. If recombination does occur, new replication-deficient vectors will be produced in which just one of the regions mentioned has been reintroduced.

5.3 Chance of RCA formation in HD AdV production systems

No RCA can be formed during the production of HD vectors by means of homologous recombination, because the vector genome consists only of the ITRs and the packaging signal. However, the probability of RCA being generated from the helper vector cannot be ruled out. First-generation ΔE1/ΔE3 AdV vectors are usually used as helper vectors, although helper vectors with only the E1 region or the E1 and E2 regions removed can also be used. The chance of RCA being generated from the helper vector depends on the helper vector used in combination with the production cell line, as described in section 5.1 (Chance of RCA formation during the production of first-generation AdV vectors).

6. Considerations

Environmental risk assessments of clinical trials focus primarily on whether or not people or animals not taking part in the trial (third parties) could be infected with the AdV vector or sequences derived from it and suffer adverse effects as a consequence. The potential environmental risks are related to the pathogenicity of the vector, the possible spread of the vector in the environment and the possible formation of recombinant viruses through recombination during the production of the vectors or in the recipient. This generic advice describes the conditions applying to replication-deficient AdV vectors based on adenoviruses of human origin or from non-human primates. The factors relevant to a generic environmental risk assessment of these vectors are discussed below.

6.1 Pathogenicity of replication-deficient adenoviral vectors

Replication-deficient AdV vectors are mostly used as vaccines against infectious diseases, but can also be used as treatments for cancer, cardiovascular and genetic diseases.[54] First and second-generation AdV vectors, like the HD vectors, are replication-deficient because of the deletion of the E1 region and are not able to replicate independently. With one or more additional deletions the vectors can be further attenuated. This applies in particular to second-generation AdV vectors and the HD vectors, but also to first-generation vectors when not only the E1 but also the E3 region is deleted from the genome.

6.1.1 Possible adverse effects

Much knowledge has been gained worldwide about AdV vectors in gene therapy studies. The method used to administer the vector may influence the reported adverse effects. Various vaccine studies have indicated that AdV vectors are generally well tolerated.[e.g.,55,56,57] Local administration of replication-deficient vectors is generally associated with relatively few and often mild self-limiting clinical symptoms, such as fever and muscle and joint pain. Systemic administration of replication-deficient AdV vectors can in some cases lead to more severe side effects.[19]

To date there has only been one known case in which gene therapy with an AdV vector had a fatal outcome. This was an 18 year old patient with ornithine transcarbamylase (OTC) deficiency who was systemically administered with a replication-deficient AdV vector (HAdV5 with a deletion in the E1 and E4 regions). The patient developed a systemic inflammatory response after infusion with the AdV vector into the bloodstream, followed by multiple organ failure, and died 98 hours after administration of the vector.[58] It is not entirely clear why this patient, unlike others, suffered an extreme reaction to the vector. Possible causes are a genetic predisposition to an overactive immune system or an increased immune response caused by a previous exposure to the adenovirus on which the vector was based.[59]

In addition, two clinical trials that investigated the use of HAdV5 vectors as vaccines against HIV-1 infections found that the chance of contracting HIV-1 was higher in vaccinated men who had built up immunity to HAdV5 before the study.[60,61] It is hypothesised that this increased chance of infection was caused by the immune reaction to the HAdV5 vector, in part because the specific CD4 T cells generated by the HAdV5 vector have an increased sensitivity to HIV.[62,63,64]

At the time of writing this generic advice, the use of AstraZeneca's COVID-19 vaccine was temporarily suspended in the Netherlands and in several other European countries following reports of the combination of severe symptoms of thrombosis and reduced platelet counts in adults following vaccination.[65] This vaccine is based on the chimpanzee AdV ChAdV Y25 and expresses the Spike gene of SARS-CoV-2. In the meantime, the Medicines Evaluation Board (CBG) and the EMA have concluded that a possible link between these very rare symptoms (at the time of writing 25 cases among more than 20 million vaccinated people in Europe)[66] and the AdV vaccine used cannot be ruled out, but that the chance of these symptoms occurring is extremely small and that the benefits in the form of protection against COVID-19 far outweigh the risks.[67] In the meantime, vaccination with this vaccine has been resumed.[68]

Besides the properties of the vector itself, the risk assessment of recombinant AdV vectors should take into account the possible adverse effects of the inserted gene product, such as those of transgenes associated with virulence, toxins or cytotoxins and the restoration of lost functions. COGEM notes that any health risks or adverse effects of AdV vectors in the first instance present a risk to the recipient. Whether or not these effects can also occur in third parties depends on the degree of spread and third party exposure to the AdV vectors (see section 6.2).

6.1.2 Integration of the vector genome into the host genome

The adenovirus genome does not contain sequences that enable active integration into the host genome.[69] However, in rare cases, passive integration of the adenoviral genome into the host genome can occur.[70]

This has been reported in hamsters inoculated with human adenoviruses (particularly HAdV12, but also HAdV18 and 31).[71] In most cases (70–90%), integration into the genome led to tumour formation,[72,73] but only when there was no lytic infection, as is usual in infections with wild-type adenoviruses. Hamster cells are non-permissive for HAdV12; replication of the HAdV12 genome is blocked, which allows the infected cells to survive for longer.[71] Various in *vitro* studies show that under experimental conditions integration of the replication-deficient vector genome can occur at a frequency of 0.0001–1% in various host cells.[74,75] In animal studies in which a replication-deficient AdV vector was intravenously administered and vector sequences were detected in mice ovaries and testes, in the next generation no evidence was found of transfer by chromosomal integration in germ cells.[76] Neither was any evidence found of germ cell integration when the AdV vector was injected into the testis or epididymis of the mouse.[77]

AdV vectors have been widely used in clinical trials worldwide for a long time. As far as is known, in clinical trials there have never been any reports of tumours arising from genome integration of AdV vectors.

6.2 Spread of the AdV vector and third party exposure

6.2.1 Biodistribution and shedding of AdV vectors

The level of excretion after administration of replication-deficient AdV vectors is generally limited due to the replication-deficient properties of the vectors. A review article published in 2007 which looked at the excretion of various vectors in clinical studies found that in 21 of the 50 published studies no excretion of replication-deficient AdV vectors was observed for various administration routes (e.g. intratumour, intranasal/inhalation, intramuscular, intrapleural).[78] The biodistribution of vector sequences depends on the administration route; some clinical studies have shown that when administered via the nose or by inhalation, vector sequences were briefly present in saliva and nasopharyngeal fluid, and that after intratumour injection vector sequences can in some cases be found in blood and blood-related products.[78] The long-term presence of vector sequences has been reported in treatments with an AdV vector via aerosol (inhalation) or nasal administration (21 days)[79] and intratumour administration in a lung tumour (90 days).[80,81] However, COGEM notes that when vector DNA is found in bodily fluids or tissues, for example by PCR analysis, this does not necessarily mean that infectious vector particles are present.

In several vaccination studies with replication-deficient HAdV5, HAdV26 and HAdV35 vectors used as potential vaccines against various diseases, no shedding of the vectors in respiratory samples or urine was observed.[82,83,84,85] In another study investigating the safety of different ΔE1/ΔE3 vectors in 90 subjects via different administration routes, 1 of 1,685 samples (pharyngeal tissue) tested positive for the presence of the AdV vector two days after nasal administration.[86]

COGEM is of the opinion that shedding of AdV vectors will be very limited (and short-lived) or non-existent. AdV vectors shed to the environment are biologically contained and will not spread further. If

the transgene or transgene product does not contribute to restoration of attenuation or complement the replication-incompetence of the vector, COGEM considers the chance of further spread of AdV vectors to humans or animals as a result of possible shedding to be negligible. In addition, COGEM points out that observing standard hygiene measures both inside and outside the hospital will further reduce the chance of third party exposure. COGEM further points out that if methods of administration are used or developed that do involve a risk of third party exposure during administration or as a result of subsequent shedding, additional measures should be taken to reduce this risk. At the same time, COGEM notes that third party exposure to vector particles will be considerably less than the initial dose administered to the test subject.

6.2.2 Formation of RCA

During the production of a viral vector or after its administration to the patient, the vector could be mobilised by complementation or recombination, or replication-competent viruses could be formed that can spread further.

Possibility of RCA formation during production

During the production of replication-deficient AdV vectors and HD vectors there is a chance that RCA will be formed by homologous recombination, as described in section 5 (RCA formation during the production of replication-deficient AdV vector). COGEM is of the opinion that the chance of RCA formation is negligible during the production of a first-generation AdV vector or an HD vector using a (helper) vector/cell line combination without sequence similarity in the E1 region, or during the production of a second-generation AdV vector. If the formation of RCA during production cannot be ruled out on theoretical grounds, the presence of RCA in the clinical product can be ruled out by performing a suitably validated RCA test.

Possibility of RCA formation in test subjects

When a replication-deficient AdV vector is administered to a person, it is possible that a cell could be simultaneously infected with the vector and a related wild-type adenovirus. If coinfection does occur, the lost functions in the vector can be restored by recombination and RCA could be produced. The chance of this occurring depends on the AdV vector used.

For second-generation AdV vectors and HD vectors the chance of RCA being formed by recombination with a wild-type adenovirus is negligible, because these vectors lack large parts of the viral genome. For first-generation AdV vectors, recombination may lead to restoration of the E1 region, resulting in a replication-competent vector. If only the E1 region in the vector is deleted, this will lead to a replication-competent virus with properties comparable to a wild-type adenovirus. If the vector also has a deletion in the E3 region, restoration of the E1 region will result in an attenuated virus. As far as is known, adenoviruses with a deletion in the E3 region have never been found under natural conditions, so it is assumed that viruses with this deletion cannot survive in the environment.[3] If the E3 region of a ΔE1/ΔE3 vector is restored by recombination, the vector will remain replication-deficient due to the

absence of the E1 region. In theory it is possible that multiple recombination events with a hybrid AdV vector will result in the formation of a virus similar to the wild-type adenovirus.

COGEM is of the opinion that the resulting recombinants will not have a higher pathogenicity or fitness than the wild-type virus that has infected the test subject. Moreover, certain administration routes used for the replication-deficient vector can further reduce the chance of coinfection. Wild-type adenoviruses replicate mainly in the respiratory tract, although in some cases the gastrointestinal and urinary tracts may also be infected.[87] When administering a replication-deficient AdV vector by the intramuscular route, therefore, the chance that the same cell will be infected by a wild-type virus as well as the vector is negligible.

If use is made of vector backbones composed of different adenoviruses, RCA formation will lead to the creation of a hybrid virus particle. As all adenoviruses have been assigned to pathogenicity class 2, COGEM has no reason to assume that this will be any different for hybrid adenoviruses and is of the opinion that the pathogenicity of this hybrid RCA will be similar to that of the parent organisms.

Besides recombination, complementation of AdV vectors may occur in the presence of wild-type adenoviruses or other viruses present in the cell at the same time as the vector. (Trans)complementation of proteins with a comparable function as the adenoviral E1 proteins can temporarily restore the possibility of replication of the vector. COGEM is of the opinion that complementation of replication-deficient AdV vectors cannot be ruled out and could lead to increased shedding of the vector, but that this will only occur for a limited period. The AdV vectors shed after complementation are still replication-deficient and cannot spread further.

6.3 Sub-conclusions

Based on the above analysis, COGEM comes to the following conclusions:

- Incidentally observed adverse effects following administration of AdV vectors concern mainly the patient or test subject. Whether or not these effects can also occur in third parties depends on the degree of spread and level of third party exposure to these vectors.
- Replication-deficient AdV vectors in which at least the E1 region has been deleted are attenuated and biologically contained, irrespective of the AdV species or (sero)type from which the vector is derived.
- Shedding of AdV vectors is very limited and further spread is not possible because they are biologically contained.
- Should an AdV vector combine with a wild-type adenovirus, or if an AdV vector is complemented, the risks to human health and the environment will be negligible.

7. Molecular characterisation of the GMO

For the environmental risk assessment it is important that the identity of the GMO has been confirmed and that the intended mutations have been made. In 2013 COGEM issued a generic advice on the genetic characterisation of GMOs for clinical applications.[88] Before replication-deficient AdV vectors may be

used as clinical applications, the identity of the final clinical product must first be determined and the sequence of the vector genome must correspond to the intended sequence. Any sequence deviations must not influence the outcome of the environmental risk assessment.

8. Advice

Based on the above, COGEM concludes that it is possible to prepare a generic environmental risk assessment for clinical applications with AdV vectors derived from adenoviruses of human origin or from non-human primates and in which at least the E1 region has been deleted. COGEM is of the opinion that these vectors are attenuated and biologically contained and are therefore not able to spread in the environment. COGEM is of the opinion that the risks to human health and the environment of clinical trials with replication-deficient AdV vectors are negligible, provided a number of conditions are met. In such trials COGEM advises adhering to the following conditions:

- The identity of the final clinical product must first be determined and the sequence of the vector genome must correspond to the intended sequence. Any sequence deviations must not influence the outcome of the environmental risk assessment.
- The transgene used does not code for a gene product that contributes to restoration of the attenuated properties or complements the replication-deficient property of the vector.
- The final clinical product does not contain RCA. If the formation of RCA during production of an AdV vector cannot be ruled out due to sequence overlap between the AdV vector and the production cell line used, the presence of RCA in the clinical batch should be ruled out by performing a validated RCA test.

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