An inventory of shedding data from clinical gene therapy trials

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Abstract

Viruses are the most commonly used vectors for clinical gene therapy. The risk of dissemination of a viral vector into the environment via excreta from the treated patient, a phenomenon called shedding, is a major safety concern for the environment. Despite the significant number of clinical gene therapy trials that have been conducted worldwide, there is currently no overview of actual shedding data available. In this article, an inventory of shedding data obtained from a total of 100 publications on clinical gene therapy trials using retroviral, adenoviral, adeno-associated viral and pox viral vectors is presented. In addition, the experimental set-up for shedding analysis including the assays used and biological materials tested is summarized. The collected data based on the analysis of 1619 patients in total demonstrate that shedding of these vectors occurs in practice, mainly determined by the type of vector and the route of vector administration. Due to the use of non-quantitative assays, the lack of information on assay sensitivity in most publications, and the fact that assay sensitivity is expressed in various ways, general conclusions cannot be made as to the level of vector shedding. The evaluation of the potential impact and consequences of the observations is complicated by the high degree of variety in the experimental design of shedding analysis between trials. This inventory can be supportive to clinical gene therapy investigators for the establishment of an evidence-based risk assessment to be included in a clinical protocol application, as well as to national regulatory authorities for the ongoing development of regulatory guidelines regarding gene therapy. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords shedding; clinical trial; retrovirus; adenovirus; adeno-associated virus; poxvirus; literature inventory

Introduction

Until now, over 1000 clinical gene therapy trials have been conducted worldwide. The majority of these trials aim at cancer treatment and to a lesser extent at the restoration of a genetic defect. In about 69% of all clinical gene therapy studies a vector of viral origin is being used, predominantly derived from adenovirus and retrovirus and to a lesser extent from adeno-associated virus (AAV), poxvirus like vaccinia virus and canary pox virus (http://www.wiley.co.uk/genmed/clinical/, overview 2006). A major environmental safety concern for the use of a vector of viral origin for gene therapy is the potential leakage of the vector into the environment via excreta from the patient. This phenomenon is called shedding. Depending on national regulations concerning the use of genetically modified organisms, shedding is an important issue in the regulatory application procedure for a clinical gene therapy protocol. If gene therapy is considered as deliberate release of a genetically modified organism into the environment, the
clinical investigator will have to perform an environmental risk assessment regarding the likeliness of shedding and its possible consequences. Historical data on shedding of a comparable vector would be of great benefit for evidence-based risk assessment. However, an extensive inventory of shedding data from clinical studies is currently not available. We have performed a literature study in order to generate an overview of shedding data from gene therapy trials that have tested the most commonly used viral vectors, i.e., retroviral, adenoviral, AAV and pox viral vectors. This overview gives an insight in the occurrence of shedding of these vectors in clinical practice and can offer support for environmental risk assessment in future clinical gene therapy applications.

Methods

Definition of shedding

In this project, shedding is defined as the dissemination of a gene therapy vector in any form into the environment, which includes contamination of other persons, through excreta from the treated subject or patient. Urine, faeces, sweat, saliva, nasopharyngeal fluids (including nasal and pharyngeal swabs and bronchoalveolar lavages), skin and semen were considered excreta. In trials using a route of administration other than injection into the circulation, blood and related products like serum, plasma and peripheral blood mononuclear cells (PBMCs) were taken into consideration as well. In general, these biological materials are not considered true excreta since they do not shed into the environment spontaneously. Nevertheless, in trials using local administration, dissemination of the vector into the circulation may turn blood into an unexpected potential source for contamination of hospital personnel involved in clinical monitoring or persons in the close environment of the treated patient. Therefore, blood and related products are also considered as excreta in trials using local administration.

The occurrence of replication-competent virus in excreta after gene therapy with a retroviral or replication-deficient adenoviral vector was considered as a special form of shedding. Replication-competent virus can either be present in the gene therapy product or generated in the patient through recombination with wild-type virus. Shedding of this vector-derived product can be a risk to the environment as well. For this specific form of shedding, blood and related products were included in the analysis regardless of the route of administration.

Literature search

PubMed was searched for articles on clinical gene therapy trials published until 31 July 2006. A limit was set on ‘Clinical trial’ as publication type and the type of virus was used as a keyword. Wildcards were used to cover as many publications as possible (e.g. ‘adenovir*’ as the keyword for adenovirus). Publications on clinical gene therapy trials, including therapeutic vaccination studies, were selected and screened for reporting on shedding analysis. Adenoviral vectors were split into two groups, namely replication-deficient adenoviral vectors and conditionally replicating adenoviral (CRAd) vectors. Figure 1 show the results of the literature search presented per vector. Overall, 260 relevant publications were selected and analyzed. Shedding analysis was reported in 102 (39%) of the selected publications. For retroviral vectors, details on shedding analysis were included in 27 (37%) out of 73 publications [1–27]. These articles were subdivided into 16 publications on in vivo retroviral gene therapy using retroviral vector-producing cells or direct administration of the retroviral vector [3,7,8,13–21,23,25–27] and 11 publications on ex vivo retroviral gene therapy treating patients with cells that had been transduced ex vivo with the retroviral vector [1,2,4–6,9–12,22,24]. For replication-deficient adenoviral vectors, 106 publications were found, of which 52 (49%) described shedding analysis. One article used as a publication on a retroviral vector was also used as a publication on a replication-deficient adenoviral vector [20]. Two publications did not provide the outcome of the analysis, resulting in a final number of 50 publications used for further analysis of shedding data [20,28–76]. For CRAd vectors, 11 (44%) out of 25 publications contained information on shedding analysis [77–87]. Five of the other publications described the testing for the occurrence of the vector in blood and related products only, while the CRAd vector was administered directly into the circulation. For this route of administration, blood was not considered as a source for shedding (see above) and therefore these articles were not included in the list of selected publications. For AAV vectors, 9 publications were found, of which 7 (78%) reported on shedding analysis [88–94]. Shedding analysis was described in 5 (11%) out of 47 publications on pox viral vectors, which were all on vaccinia vectors [95–99]. Two of these publications described the clinical testing of a replication-competent vaccinia vector [95,96]. Studies on a replication-deficient vaccinia vector and a replication-restricted vaccinia vector were reported in one [98] and two [97,99] publications, respectively. In total, 100 publications have been used to make an inventory of shedding analyses and data.

In some publications, especially on trials using a retroviral vector, the generation of an antibody response against the vector was used as a parameter for shedding. We have not included this type of analysis and have only regarded a direct measurement of viral genomic sequences, proteins or infectious particles as shedding analysis.

Results

Shedding assays

For shedding analysis, various types of assays or a combination thereof were found to be used. In general,
Figure 1. Publications on clinical gene therapy trials using a viral vector with information on shedding analysis. PubMed was screened for articles published until 31 July 2006 on clinical gene therapy trials using a retroviral vector (A), replication-deficient adenoviral vector (B), CRAd vector (C), AAV vector (D), or pox viral vector (E). The number of publications that have (solid bars) and have not (open bars) reported on shedding analysis is indicated. There is one article that has been used both as a publication on a retroviral vector and as a publication on a replication-deficient adenoviral vector [20].

Analysis consisted of the assessment of vector genomic sequences by non-quantitative or quantitative polymerase chain reaction (PCR) and/or of infectious viral particles by a biological assay. The PCR specifically measured vector DNA through vector-specific primers in most cases.

The selected publications on ex vivo retroviral gene therapy all reported on the measurement of RCR only by either a PCR or a biological assay [1,2,4–6,9–12,22,24]. For in vivo retroviral gene therapy, shedding of the vector itself was in all cases assessed by PCR [3,7,8,13–21,23,25–27]. Biological assays such as the marker rescue assay and the PG4S+L− assay were used for the analysis of only replication-competent retrovirus (RCR).

For replication-deficient adenoviral vectors, an enzyme-linked immunosorbent assay (ELISA) for the detection of adenoviral proteins was used instead of PCR in some publications (Table 1) [29,32,49,54,58,59]. In about 40% of the cases, the shedding analysis was performed by a combination of two assays, primarily PCR and a biological assay. In 13 publications, this combination was used for the confirmation of a positive result, meaning that a
positive signal in a biological assay was verified by PCR, and vice versa. Infections were mainly measured by a selective biological assay using a combination of 293 cells that enable replication of this type of vector and A549 cells that are not permissive to a replication-deficient adenoviral vector. In some publications, a flow cytometry based biological assay was described for this purpose. Replication-competent adenovirus (RCA) was assessed in a biological assay using cells that were insensitive to the replication-deficient adenoviral vector, such as A549 cells. In two publications, the assays used for shedding analysis were not specified, although test results were included.

In all publications on CRAd vectors, PCR was used for shedding analysis and in five cases this test was combined with a virus culture. Three out of these five publications reported that the two shedding assays were used as a combination for verification of a positive result.

Shedding of AAV vector was assessed either by PCR or by a biological assay. In the biological assay, samples were cultured in adenovirus-infected cells that express AAV rep protein and allow AAV vector replication, followed by PCR in the cell lysate for the detection of AAV genomes. In three publications, both PCR and a biological assay were used in an independent way. The choice of assay depended on the sample to be tested. PCR was used for analysis of blood and related products and a biological assay for other excreta.

In all publications for pox viral vectors, shedding was assessed by virus culture using for instance Vero cells. In three publications describing both a PCR and a biological assay for shedding analysis, the two tests were used for verification of positive results.

### Table 1. Types of assays used for shedding analysis

<table>
<thead>
<tr>
<th>Assay Type</th>
<th>Void vector (n = 27)</th>
<th>Repl-def Ad (n = 52)</th>
<th>CRAd (n = 11)</th>
<th>AAV (n = 7)</th>
<th>Poxvirus (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>qPCR</td>
<td>23 (85%)</td>
<td>31 (60%)</td>
<td>11 (100%)</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Non-qPCR</td>
<td>14 (13%)</td>
<td>5 (5%)</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Unknown</td>
<td>6 (2%)</td>
<td>1 (2%)</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Biological assay</td>
<td>27 (26%)</td>
<td>35 (67%)</td>
<td>5 (31%)</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>ELISA</td>
<td>0 (0%)</td>
<td>9 (17%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Unknown</td>
<td>2 (7%)</td>
<td>2 (4%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The number of publications using a certain type of assay is indicated. Percentages are based on the total number of publications per vector type. For PCR, a subdivision has been made into quantitative PCR (qPCR), non-quantitative PCR (non-qPCR) or 'unknown' if the publication provides no details. Furthermore, the number of assay types used for shedding analysis is indicated for the publications analyzed.

For retroviral and replication-deficient adenoviral vectors, two publications did not provide details on the type of shedding assays used.

*Biological assays were only applied for the assessment of RCR.

### Biological samples used for shedding analysis

Table 2 presents an overview of biological samples tested for vector shedding. In general for the four viral vectors, it can be stated that urine and blood and related products, in the case of local administration and analysis of replication-competent virus, are the most commonly tested biological samples. The choice of other biological samples appears to be related to the way of administration of the vector. For instance, skin samples are primarily analyzed in trials using intradermal injection, and nasopharyngeal fluids in trials using inhalation or intranasal administration.

A notable observation is that for replication-deficient adenoviral vectors shedding is analyzed in a wide variety of biological samples, even when the vector is administered locally, which is the case in the majority of the publications. Blood and related products, urine, faeces and nasopharyngeal swabs are the most commonly tested samples for this type of vector. In contrast, for CRAd vectors blood and related products are the main samples.

### Table 2. Biological samples used for shedding analysis

<table>
<thead>
<tr>
<th>Vector</th>
<th>Administration</th>
<th>Biological samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retrovirus in vivo therapy (16 publications)</td>
<td>ip (2), it (13), iv (1)</td>
<td>blood and related products (all publications) faeces (1), saliva (1), semen (1), skin (1), urine (2)</td>
</tr>
<tr>
<td>ex vivo therapy (11 publications)</td>
<td>id (2), it (1), iv (8)</td>
<td>blood and related products (all publications)</td>
</tr>
<tr>
<td>Replication-deficient adenovirus (50 publications)</td>
<td>ic (2), im (2), inh/in (9), ip (3), it (26), ivi (2), other* (6)</td>
<td>blood and related products (35), faeces (23), nasopharyngeal fluids (26), saliva (15), semen (2), skin (1), urine (44)</td>
</tr>
<tr>
<td>CRAd (11 publications)</td>
<td>id (1), it (8), it + iv (1), it/ip (1)</td>
<td>blood and related products (9), skin (1), urine (3)</td>
</tr>
<tr>
<td>AAV (7 publications)</td>
<td>ia (1), im (1), inh/in (5)</td>
<td>blood and related products (5), faeces (4), nasopharyngeal fluids (3), saliva (4), semen (2), urine (5)</td>
</tr>
<tr>
<td>Pox virus (5 publications)</td>
<td>id (2), im (2), it (1)</td>
<td>blood and related products (3), faeces (1), nasopharyngeal fluids (2), saliva (1), skin (2), urine (3)</td>
</tr>
</tbody>
</table>

The number of publications is indicated in parentheses. Blood and related products include serum, plasma and peripheral blood mononuclear cells. Nasopharyngeal fluids include nasal and pharyngeal swabs and bronchoalveolar lavages.

Abbreviations: ic; intracoronary, id; intradermal, im; intramuscular, inh/in; inhalation/intranasal, ip; intraperitoneal, it; intratumoral, iv; intravenous, ivi; intravitreal

*Other routes of administration include intraarterial (1), intradural (1), intramyocardial (1), intrapleural (2) and intravenous (1)
used for shedding analysis. Furthermore, for retroviral vectors applied by ex vivo gene therapy the shedding analysis concentrated on the occurrence of replication-competent retrovirus (RCR) in blood and related products. The number of publications on AAV and pox viral vectors is limited, but there is a trend that shedding analysis is not restricted to blood and related products only. For AAV, five out of seven publications focused on gene therapy for cystic fibrosis through inhalation or intranasal administration [88,89,92–94]. In these trials, shedding analysis was performed in samples derived from the site of administration, such as saliva and nasal and pharyngeal swabs. Overall, only five of the 100 analyzed publications have reported on shedding analysis in semen [15,37,43,90,91].

**Time schedule for shedding analysis**

There is substantial variability in the timeframe of shedding analysis (data not shown) and therefore publications are difficult to compare in this respect. In general, for retroviral vectors analysis of vector sequences was performed in the first month after administration. In contrast, analysis of RCR was on average continued on a regular basis till 1 year after the treatment or beyond. In most publications both for replication-deficient adenoviral vectors and CRAd vectors, the shedding analysis was performed in the first month after administration. For AAV vectors, shedding was studied in the first weeks after administration and in one study was continued for 4 months [91]. For pox viral vectors, the shedding analysis appeared to be limited to the first 2 weeks only and the longest period of shedding analysis continued till 21 days after administration [95].

**Shedding data**

Overall, shedding data from 1619 treated patients have been collected (Table 3). It should be noted that the total number of included patients is higher, since in some publications a selection of patients has been tested for shedding. Although quantitative shedding data are described and information on assay sensitivity is provided in a number of the publications, the observed level of shedding is not discussed in this overview. Comparison of quantitative shedding data is hampered by the highly variable way quantity is expressed. For instance, the outcome of a PCR was defined as copy number, particles, particle-forming units (pfu) or positive cells, in most case related to a genomic DNA quantity, cell number or sample volume. As such, general conclusions cannot be made as to the level of vector shedding. Since it is not possible to discuss the shedding data for each individual publication in this overview, a summary of the observations is presented per vector type. The publications in which the assay sensitivity is described and/or quantitative shedding data are reported are listed as well.

**Retroviral vectors**

All selected publications on ex vivo retroviral gene therapy trials focused on the occurrence of RCR in blood or related products only, as mentioned before. No RCR was detected in any of the 103 patients tested. Also for in vivo retroviral gene therapy, no RCR was observed in a total of 342 patients tested. In 10 out of 16 publications on in vivo retroviral gene therapy, the presence of vector in blood (primarily PBMCs) was demonstrated by PCR after intratumoral gene therapy for brain tumours [8,14,16,17,21,27], melanoma [3,7], and breast tumours [3], and intraperitoneal gene therapy for ovarian cancer [25,26]. Specifically, in 6 out of 8 publications describing gene therapy for brain tumours by intratumoral administration, vector sequences were found in PBMCs [8,14,16,17,21,27]. The duration of shedding varied from 1 to 28 days after administration. In none of these publications was the finding of vector genomic material confirmed as infectious viral particles. In one publication, semen from 13 patients was tested for the presence of vector at regular intervals up to week 53 after intravenous vector administration for the treatment of haemophilia A [15]. For one patient, a positive semen sample, being a 1 out of 10 replicate, was obtained at week 9. Since all previous and subsequent semen samples were negative, it could not be determined if this positive signal was present in the motile sperm fraction. Overall, in 10 out of the 27 publications either quantitative shedding data were reported or information on the assay sensitivity was provided [2–4,7,8,12,15,17,19,20].

**Replication-deficient adenoviral vectors**

In 21 out of 50 publications (42%), shedding of vector was not observed after intratumoral [20,32,34,46,50,52,60, 61,67], intranasal or inhalation [31,39,45,71], intracoronary [37,42], intramyocardial [57], intravitreal [73],
intraarterial [51], intrapleural [62,63] or intramuscular [75] administration. In addition, in one publication on gene therapy by intravitreous administration only RCA was measured with a negative outcome [72]. Five out of nine publications that did not find vector shedding in blood, urine or other excreta after intratumoral administration are related to the treatment of brain tumours [20,34,50,61,67]. A sixth publication on the same disease did show some shedding in plasma at one time point in 2 out of 7 patients [44].

The remaining 29 publications (58%) reported on shedding of vector DNA or infectious particles in various excreta, primarily depending on the route and site of administration and the time of analysis. For instance, saliva and nasopharyngeal fluids from some patients treated with a vector by intranasal administration or via inhalation were found to contain vector in a number of studies [28,47,55]. In a substantial number of publications (17 out of 26) describing intratumoral gene therapy in patients with various types of cancer shedding was demonstrated, primarily in blood and related products [30,33,35,36,43,44,48,49,53,54,58,59,64–66,68,74]. In general, shedding in blood was short lasting, peaking during the first hours and disappearing a few days after administration. In one of these studies, vector sequences were found in urine from the majority of treated patients up to day 32 after injection of the vector in prostate tumour cells [43]. The longest duration of shedding was reported in publications on the treatment of cystic fibrosis via inhalation [28] or of lung cancer using intrabronchial [38] or intratumoral administration [68]. After a single treatment, nasopharyngeal fluids like bronchoalveolar lavage, nasal and pharyngeal swabs and saliva were reported to be positive for vector sequences up to 21 [28], 30 [68] and even 90 days [38]. Two publications reported on the analysis of semen from 12 patients with angina pectoris 8 weeks after intracoronary administration [37] and from one patient with prostate cancer at day 14 after injection in a prostate tumour [43], respectively. The latter patient appeared to be positive for the vector based on PCR analysis.

Only a minority of the publications reported on the verification of a positive shedding finding. In 7 out of 8 publications, a positive PCR signal was confirmed as being derived from infectious adenoviral vector particles [30,35,36,47,53,68,74]. Eleven out of 50 publications on replication-deficient adenoviral vectors specifically studied the occurrence of RCA [20,30,31,38,39,50,53,57,64,65,72]. In none of the 201 patients tested could the presence of RCA be demonstrated.

In 18 out of the 50 publications either quantitative shedding data were reported or information on the assay sensitivity was provided [20,30,32,37,38,43–45,53,54,64–67,70,71,75,76]. Taking the definition of shedding into consideration, the occurrence of the vector in the environment of the patient is an interesting parameter. Four publications were found describing shedding analysis in blood, faeces or throat swabs obtained from health care personnel who came into close contact with the patient [30,36,38,68]. Interestingly, in none of the individuals studied (up to 54 in one publication [68]) could the presence of the vector or RCA be demonstrated.

**CRAv vectors**

Three out of 11 publications describing an intratumoral application or an intraperitoneal administration reported a negative outcome for vector shedding in blood, urine or skin [81,84,87]. In the other 8 publications, the occurrence of vector DNA was demonstrated in blood by PCR after intratumoral administration of the CRAv vector [77–80,82,83,85,86]. The duration of shedding in blood ranged from a few hours to 76 days after administration. In two publications shedding results pointed to viral replication, demonstrated by a second peak of circulating viral genome [77,80]. In two publications it was studied if the presence of vector DNA in blood could be linked to shedding of infectious viral particles. No infectious viral particles were demonstrated [78,79]. In contrast, shedding of infectious vector particles was demonstrated in urine up to day 8 after vector injection in a prostate tumour [77].

In seven publications either quantitative shedding data were reported or information on the assay sensitivity was provided [77–80,83,85,86].

**AAV vectors**

Four of the five publications related to gene therapy for cystic fibrosis reported on shedding in nasopharyngeal samples and saliva during the first days after inhalation or intranasal administration [88,89,92,93]. Shedding in blood was rarely observed [88]. In the other two publications, the outcome of two clinical trials on gene therapy for haemophilia B was described. Intramuscular administration resulted in shedding of the AAV vector as measured by PCR in saliva and serum up to 24 and 48 h after injection, respectively, and no vector was observed in semen obtained after about 2 months [90]. When patients were treated with the same vector by infusion through the hepatic artery, dose-dependent shedding in urine was found during the first post-treatment week [91]. In 6 out of these 7 treated patients, vector DNA was found in semen up to 16 weeks after therapy. In one patient the vector was present in the seminal fluid and not in motile sperm [91].

In six publications either quantitative shedding data were reported or information on the assay sensitivity was provided [88–93].

**Pox viral vectors**

In all five publications studied, the replication-competent [95,96], replication-restricted [97,99], or replication-deficient [98] vaccinia vector was administered locally. No shedding was observed in blood, urine, faeces, saliva, nasopharyngeal samples and skin in four publications.
One publication reported on the presence of live vaccinia virus in the detached scab from all eight treated patients after intradermal administration of a replication-competent vaccinia vector, but not in swabs taken from outside the injection site [96]. This was confirmed as the vaccinia vector by PCR. In this same publication, testing of wound dressings, hospital furniture and bed linen and air samples for the presence of vector was described [96]. Live vaccinia virus was only demonstrated in used dressings covering the injection site. In two publications information on the assay sensitivity was provided [96,99].

Discussion

This article presents an inventory of shedding data from publications on clinical gene therapy trials using retroviral, adenoviral, AAV and pox viral vectors. The literature search strategy was validated by a random test using the GEMCRIS database of American gene therapy trials. It appeared that the literature search strategy covered about 90% of the articles published on these clinical studies. Thus, we believe that a representative selection of relevant publications has been obtained and evaluated in this study.

Shedding analysis is reported in less than half (39%) of the publications found in our literature search. Data on shedding are not reported in the majority of publications on clinical gene therapy trials. In addition, the requirements for monitoring on the occurrence of shedding depend on national gene therapy regulations that are continuously adapted to previous experience and future development. Thus, the extent of availability of shedding data will vary between trials. In the articles that were found to report on shedding analysis, information on the set-up of shedding analysis as well as on the shedding data is often limited and the experimental design of shedding analysis varies significantly between trials. These observations suggest that shedding is not a prominent part of a report on the clinical outcome of gene therapy, while from the inventory it can be concluded that shedding of viral vectors is a common phenomenon for clinical gene therapy. Shedding mainly depends on the type of vector and the route of administration. For instance, in 36 out of 61 publications on adenoviral vectors, positive shedding results have been reported, in contrast to 1 out of 5 publications on pox viral vectors, while both vector types primarily were administered locally. Positive samples are often derived from excreta obtained close from the site of administration, such as nasopharyngeal samples and saliva after inhalation or intranasal administration [28,38,55,88,92], skin or scabs after intradermal administration [40,96], or urine after local administration in a prostate tumour [43,77]. It is of interest to note that local administration of retroviral and adenoviral vectors, primarily by intratumoral injection, in general is associated with shedding in blood. An exception is the finding that adenoviral vector shedding hardly occurs after injection in a brain tumour [20,34,44,50,61,67], suggesting that leakage of this vector type from this specific part of the body is an unlikely event. In contrast, shedding of retroviral vector to PBMCs after local administration in a brain tumour was reported in 6 out of 8 publications found. This is ascribed either to leakage of the vector or vector producing cells into the blood [27] or to the migration of locally transduced lymphocytes or tumour cells to the circulation [17,21,26].

Shedding is defined as the dissemination of a gene therapy vector into the environment via excreta from the treated patient. Since blood does not meet the criteria of excreta, potential dissemination of a vector through blood is often disregarded. However, the observations reported in this overview demonstrate that a viral vector can disseminate into the circulation after local administration. For hospital personnel involved in the clinical monitoring or treatment of a patient as well as persons in the close environment of the patient, blood can thus be a potential source of contamination if released deliberately during hospital procedures or by accident, as would also be the case for blood from a patient treated by direct administration of a vector into the circulation. This aspect has not been taken into consideration in this study. It is important to note that also in these cases blood can be a potential source for vector dissemination into the environment, although this is not regarded as shedding.

The effect of the administration route on vector shedding is most clear in two trials on AAV-mediated gene therapy for haemophilia B [90,91]. After intramuscular administration, shedding of the AAV vector was primarily observed during the first 2 days in saliva and serum and not in urine. The vector could not be detected in semen obtained after about 2 months [90]. In contrast, after infusion through the hepatic artery of the same vector, shedding was observed in urine during the first week. In addition, in 6 out of the 7 treated patients, vector DNA was found in semen up to 16 weeks after therapy [91]. Based on these observations, the FDA Biological Response Modifiers Advisory Committee (currently the Cellular, Tissue and Gene Therapies Advisory Committee) decided in 2002 that a positive semen test is no indication to put a clinical trial on hold, provided that the vector does not persist in semen longer than 1 year [100]. In that case, a trial will be halted in order to study the occurrence of germ-line transmission and to anticipate on the potential consequences of such an event. Furthermore, long-term monitoring of sperm was recommended by this committee.

It is difficult to draw conclusions on the duration of shedding in relation to the vector type. There are substantial differences between the publications with respect to factors affecting shedding analysis that complicate comparison of results, including the time schedule of shedding monitoring, type of shedding assay used, excreta tested, way of administration, dosage and dosage schedule. A number of observations on CRAd vectors indicate shedding after in vivo replication, which
suggested that a replicating vector may shed for a longer period [77,80,86].

The generation of replication-competent virus in the patient is a major safety concern for replication-deficient vectors, especially for retroviral gene therapy. An important conclusion from this inventory is that until now replication-competent virus has not occurred in patients treated in vivo or ex vivo with a retroviral vector or with a replication-deficient adenoviral vector. This is based on results reported for 445 and 201 patients, respectively. Interestingly, for ex vivo retroviral gene therapy, treating a patient with cells that have been transduced with the vector ex vivo, shedding analysis only focuses on the assessment of RCR and not on the vector itself. According to investigators involved in this type of gene therapy, vector shedding is considered to be negligible by regulatory authorities due to the quality requirements regarding the absence of free vector particles in the treated cells.

Another safety issue of clinical gene therapy is spreading of a vector to the gonads, resulting in the risk of germ-line transmission. Shedding of vector in sperm can be an indication for biodistribution to the gonads. It appears that testing of semen is not a common practice in clinical gene therapy trials. In this inventory, five relevant publications are included of which three publications have reported on the occurrence of a retroviral vector [15], replication-deficient adenoviral vector [43] and an AAV vector [91] in semen. The presence of the retroviral vector in semen was explained by a false-positive result, since it concerned a 1 out of 10 replicate and previous and subsequent samples were negative [15]. The finding of adenoviral vector in the semen from one patient, which was the only sample collected [43], may be explained by the fact that the vector was injected intraprostatic and may have been excreted in semen via prostatic fluid. Notably, this sample was collected after 14 days while the sperm generation time in humans is 8 weeks [37]. Therefore, this shedding observation may not be related to biodistribution of the vector to the gonads. For the AAV vector, 6 out of 7 treated patients had positive semen samples. Based on the results of a study on one patient, it was demonstrated that the vector was present in the seminal fluid and not in the motile sperm fraction [91].

A crucial question is the relevance and implications of the positive shedding data presented in this overview. In principle, the moment of vector administration is the most critical time point in shedding since the environment is at that particular moment exposed to a high vector titer. The level of vector shed via excreta will be far lower. However, none of the publications have specifically addressed shedding analysis at this time point. It is important to note that in general positive shedding data are reported for only a part of the patients tested. In addition, shedding is often short lasting and is observed during the first 1 or 2 days after vector administration. Based on this inventory, it is hard to speculate on the level of shedding in general in the past clinical trials. In a number of the publications the assay sensitivity is defined or quantitative shedding data are reported. Nevertheless, since assay sensitivity is expressed in various ways, general conclusions as to the level of vector shedding could not be drawn. In addition, a positive PCR result points to the presence of vector DNA or RNA and is not necessarily indicative for potential harmful infectious viral particles. The occurrence of viral genomic material in excreta does in itself not pose a threat to the environment. Data on shedding of infectious particles would be of value in this respect, but availability of this type of information appears to be limited. None of the publications on ex vivo retroviral gene therapy reported on shedding analysis of infectious retroviral particles or vector genome, most likely due to the fact that the likelihood of vector shedding is considered negligible. In contrast, infectious retroviral particles can in principle be shed during in vivo retroviral gene therapy, although in the selected publications shedding analysis appears to be limited to vector genome testing by PCR only. In a limited number of publications on adenoviral vectors, PCR-positive samples were verified for the presence of viral particles using a biological assay. A number of these samples were confirmed to contain infectious particles in 7 out of 8 publications, indicating that shedding of intact adenoviral vector does occur in practice. In none of the publications on AAV vectors reporting on shedding of vector genomic material was this positive finding confirmed by a biological assay. The assessment of infectious AAV particles may be experimentally complicated by the fact that AAV is a non-cytopathic virus that depends on adenovirus for its replication. Nevertheless, in the majority of the publications excreta other than blood were tested for the presence of infectious particles only, indicating that the clinical use of a biological assay of AAV is feasible. Interestingly, for pox viral vectors the assessment of infectious particles appears to be common practice for shedding analysis and even is the first or only shedding assay carried out.

The potential impact of being exposed to an intact and infectious viral vector shed from an individual treated by gene therapy depends on the capacity of the vector to replicate and integrate as well as on the therapeutic transgene. For instance, the environmental threat imposed by shedding of a replication-deficient vector coding for a suicide gene, which has no effect in the absence of a prodrug, will be far less compared to shedding of a replication-competent vector coding for a cytotoxic gene. In addition, it is currently unknown if viral vector particles that are shed into the environment via excreta from treated patients are able to infect third parties and which critical level of shedding needs to be achieved in order to enable cross-contamination. In four publications that have reported positive shedding results, of which three publications described infectious particles in various excreta [30,36,96], tests of samples obtained from hospital personnel and furniture were all negative. However, it is most likely that the hospital safety measures taken in these studies were adequate to prevent cross-contamination. In one of these studies [96], describing
intradermal administration of a vaccinia vector, live virus was observed in wound dressing while the vector was also found in the detached scab, indicating that the presence of vector in excreta may indeed result in contamination of the environment. There are historical data on adenovirus available indicating that inhalation of a minimum of 10^5 pfu of virus is sufficient to induce a clinically apparent infection of the respiratory tract [101]. Extrapolation of these data to contamination of humans with genetically modified adenoviruses via excreta is complicated and only preclinical studies may give an indication of the level of shedding of infectious particles required to induce clinical symptoms in third parties.

The inventory of shedding data presented in this overview is part of the outcome of a project entitled ‘Inventory of shedding data and analyses: possibilities for standardization’ that has been supported by the Commission on Genetic Modification (COGEM), a Dutch advisory body to the government on the risks to human health and the environment of the production and use of genetically modified organisms. The purpose of this project was to evaluate the possibilities for standardization of shedding analysis in clinical gene therapy trials in The Netherlands. Based on discussions with national and international academic and commercial gene therapy experts as well as experts from regulatory authorities, and the inventory of shedding data presented in this overview, it has been concluded that standardized shedding analysis is not realistic due to the current lack of uniformity in shedding analysis in clinical gene therapy studies. This is explained by differences between national environmental regulations concerning gene therapy as well as the lack of regulatory guidelines on shedding analysis. The COGEM has been recommended to develop a guidance or decision tree on shedding analysis that addresses the need to perform shedding analysis, the type of excreta that should be tested, the methods that could be used for analysis and the technical requirements for the shedding assays. Critical parameters for such guidance are the type of vector to be used, the therapeutic transgene and the way of administration. By offering such guidance to the gene therapy investigator, the degree of uniformity in shedding analysis between clinical trials may be increased, which will contribute to evidence-based environmental risk assessment.

Conclusions

Based on literature data on shedding of viral vectors during clinical gene therapy as presented in this overview, the following conclusions can be made:

- the relevance and implications of shedding remain a subject for further investigation.

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