Short and long term dissemination of deletion mutants of adenovirus in permissive (cotton rat) and non-permissive (mouse) species

W. Oualikene, P. Gonin and M. Eloit*

Unité de Génométique Moléculaire, Génométique Virale, INRA, Ecole Nationale Vétérinaire d'Alfort, 94704 Maisons Alfort, France

As a first step in the evaluation of the safety of replication-defective adenoviruses used as gene transfer vectors, the dissemination of wild-type (wt) adenovirus (Ad) type 5, Ad-dl327 (deleted for the E3 gene) and Ad-gp50 (deleted for E3 and E1A and therefore replication defective) was studied in mice and cotton rats. Of each virus, 10^8 p.f.u. was inoculated, either by the intranasal or the intramuscular route, and virus isolation was undertaken after sacrifice of the animals 3 or 31 days after inoculation. E3 deletion had no significant effect on viral spread, whereas E1A deletion reduced it significantly. After intramuscular inoculation of the E3-/E1A- virus, it could be isolated only from the local lymph node. Intranasal inoculation led to a wider distribution including lungs, liver, kidneys and lymph nodes. The pattern of dissemination of the E3-/E1A- virus was the same in mice and cotton rats, providing indirect evidence of the lack of replication of this virus in this species permissive for the wt virus. However, in the case of infection with a wt strain in E3-/E1A- adenovirus-inoculated recipients, both viruses were found in lymph nodes. In this situation the risk of phenotypic complementation of the E1A gene remains to be studied further.

Adenovirus vectors are being used more and more widely for gene transfer, especially in non-dividing cells (Stratford-Perricaudet et al., 1991; Akli et al., 1993; Caillaud et al., 1993; Guzman et al., 1993; Haddada et al., 1993; Jaffe et al., 1992; Le Gal La Salle et al., 1993; Schneider & French, 1993; Ragot et al., 1993a). Vaccines based on replication-defective adenoviruses have also been developed (Eloit et al., 1990; Ragot et al., 1993b; Adam et al., 1994). Replication-competent serotypes 4 and 7 have proven safe when administered to U.S. military recruits (Cough et al., 1963; Top et al., 1971a, b). Oral types 1, 2 and 5 adenovirus vaccines were safe in volunteers during a clinical trial (Schwartz et al., 1974).

The safety of replication-defective adenovirus vectors is based on the deletion of the E1A region. This region encodes the immediate early proteins that trans-activate all other early regions, so that DNA replication in these deletion mutants is dramatically reduced. However, several concerns about the safety of these vectors remain to be studied. Apart from the clinical safety for people or animals inoculated with these viruses, which must be studied on a case-by-case basis, a major point is the risk of horizontal spread of the virus. It is obvious that for safety reasons the spread of recombinant viruses harbouring genes encoding proteins with pharmacological properties is not acceptable. In theory, this can happen if a E1A-deleted virus is mixed with a wild-type (wt) virus (in people or animals previously or subsequently infected), providing phenotypic 'trans-complementation' of the E1A gene leading to a mixed viral population, or if DNA recombination between viruses occurs, leading to a replication-competent E1A+ vector harbouring a foreign gene.

The first step towards answering this question is to determine the dissemination of the wild-type and E1A-deleted viruses in the bodies of inoculated animals, using several routes of administration. The objective is to discover which organs are possible sites for transcomplementation. Two administration routes were tested: (i) intranasal, a natural route that may also be used for respiratory tract illness gene therapy and (ii) intramuscular, used for muscular gene therapy as well as for vaccination.

Two animal adenovirus infection models have been previously developed. Cotton rats (Pacini et al., 1974), which are permissive to many human respiratory tract illnesses, and mice (Ginsberg et al., 1991), which are not or only weakly permissive to adenovirus type 5 (Ad5). In this species, a virus dose of 10^10 p.f.u. was required to reproduce a similar pathology to that described in cotton rats with 10^8 p.f.u. (pneumonia with alveolar cell infiltration; Prince et al., 1993). These two animal models were used in this study to provide information not only...
**Table 1. Virus isolation three days after inoculation**

<table>
<thead>
<tr>
<th>Organ virus titre (log&lt;sub&gt;10&lt;/sub&gt; p.f.u./g)*</th>
<th>Cotton rat</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection route</td>
<td>Liver</td>
<td>Lungs</td>
</tr>
<tr>
<td>-----------------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>i.m. Wt E3−/E1A+</td>
<td>+/+</td>
<td>−/+</td>
</tr>
<tr>
<td>i.m. Wt E3−/E1A−</td>
<td>−/+</td>
<td>−/+</td>
</tr>
<tr>
<td>i.n. Wt E3−/E1A+</td>
<td>+/+</td>
<td>7/7</td>
</tr>
<tr>
<td>i.n. Wt E3−/E1A−</td>
<td>−/+</td>
<td>7/7</td>
</tr>
<tr>
<td>Control</td>
<td>−/+</td>
<td>−/+</td>
</tr>
</tbody>
</table>

* Results are given as follows: first animal/second animal. Samples found positive at the first passage were titrated, samples positive at the second passage are indicated by (+). No samples were found to be positive on the third passage.

† Titres for nasal washings are given as log<sub>10</sub> p.f.u./ml.

Concerning human, but also animal species either permissive (like pigs) or not (like cats) to human adenoviruses.

Apart from the wt Ad5, we have included two deletion mutants: (i) Ad-d1327 (Jones & Shenk, 1978; Thimmmapaya et al., 1982) deleted from 78.5 to 84.3 map units in the non-essential E3 region, from which E1A-deleted recombinant vectors are often constructed. Ad-d1327 replicates as the wt (Andersson et al., 1985; Ginsberg et al., 1989) but provokes inflammatory injuries enhanced in magnitude due to the lack of gp19, which normally reduces the expression of major histocompatibility complex (MHC) class I antigens on infected cells surfaces. These cells are therefore not recognized by cytotoxic T cells; (ii) Ad-gp50 (Eloit et al., 1990), a recombinant adenovirus deleted for E3 and E1A which expresses gp50 of pseudorabies virus.

Cotton rats (Sigmodon hispidus) and BALB/c mice, 4 weeks of age, were obtained from IFFA-CREDO. Eight animals of each species were infected intranasally (i.n.) or intramuscularly (i.m.) in the back of the thigh with 100 μl (50 μl per nostril or 50 μl per thigh) containing 10⁴ p.f.u. of each adenovirus in MEM. Control animals were inoculated using crude cell extracts. Four animals from each group were sacrificed 3 days after inoculation, and the other four 31 days after inoculation. Various organs (spleen, liver, lungs, kidneys, brain, ileum and popliteal and inguinal lymph nodes) were removed aseptically, weighted and then used for virus isolation. Fragments for viral isolation were ground in 1 ml of MEM using an Ultra-turrax grinder. Virus isolation was then performed on the 293 cell line. The first inoculum was 200 μl of extract in MEM ranging from 1.5 to 1700 mg/ml depending upon the organ tested. The 293 cell line, an adenovirus-transformed human embryonic cell line containing the E1 region of adenovirus integrated in its genome (Graham et al., 1977), was used for adenovirus amplification and titration. These cells were maintained in MEM with 10% fetal calf serum. Cells were observed daily for 7 days. In the case of negative results, two blind passages were performed. For each passage, the virus was released from cells by three cycles of freezing and thawing. Nasal washes were obtained by inserting and twirling an Eppendorf yellow tip in each nostril while pipetting several times approximately 100 μl of MEM.

No deaths or clinical signs of illness were observed in any of the animals. Results obtained with the organs taken from animals killed 3 days after inoculation are summarized in Table 1.

In the permissive species (cotton rats) inoculated i.n. with the wt virus, each of the organs tested proved positive. Less dissemination occurred after i.m. inoculation, but the wt virus could be isolated from liver, lung, spleen, ganglia and even from nasal washes. Dissemination of Ad-d1327 was not significantly different from that of the wt virus for the two tested routes. The only clear difference was that d1327 could be recovered at high titres in each tested lymph node after i.n. inoculation, whereas only low levels of the wt virus were found. In clear contrast, the E1A/E3-deleted virus (Ad-gp50) could only be recovered from the lungs, kidney and...
ganglia after i.n. inoculation and was not isolated from any organ after i.m. inoculation.

In the non-permissive species (mouse), i.n. inoculation of either the wt, dl327 or E1A/E3 deleted-virus led to the same pattern of distribution as in the cotton rats. Nevertheless, a clear difference was that replication of E1A+ viruses was less important than in cotton rats, in which virus isolation was often made after only one passage. After i.m. inoculation of the E1A/E3-deleted virus, isolation was positive only in the local lymph node (popliteal). Both E3-deleted viruses (Ad-dl327 and Ad-gp50) could be recovered from ganglia at titres up to 10^6.1 p.f.u./g in some animals, whereas the wt virus could not be isolated from the proximal lymph node after i.m. inoculation.

Attempts to isolate viruses from the organs of animals killed 31 days after inoculation were unsuccessful except for one cotton rat inoculated i.m. with the wt virus: the ileum proved positive on the first passage (titre: 10^6.3 p.f.u./g) and the lungs were positive in the second passage.

These results show that the probability of excretion of E1A-deleted viruses is mainly a function of the route of administration. The i.m. route seems to be particularly safe, as in both mice and cotton rats, infectious virus could not be recovered except in the proximal lymph node. These results confirm those obtained in pigs, another permissive species (Betts et al., 1962), in which Ad-gp50 could not be isolated from nasal and rectal swabs taken in the days following i.m. administration of 10^8 TCID50 of Ad-gp50 (M. Adam, unpublished results). This is in contrast with the wt and dl327 viruses, which could be isolated in lungs and nasal washes after i.m. inoculation. Virus spread was the same for the wt virus and Ad-dl327, showing that the E3 deletion had no clear effect on dissemination, except, possibly, in lymph nodes from where E3-deleted mutants seemed to be isolated with ease. On the other hand, i.n. inoculation is far less safe: Ad-gp50 could be isolated from the kidneys, potentially leading to urinary excretion of the virus. Isolation from the lungs, nasal washes, popliteal nodes and liver were also positive, but most likely related to local spread of the input virus. Nevertheless, quite high titres could still be found 3 days after inoculation in the lungs and nasal cavities showing that several days in isolation are necessary for patients treated by this route of administration.

It is obvious from our results that the cotton rat is a good model for the in vivo study of the biosafety of recombinant adenoviruses, as wt virus can be isolated from all organs after i.n. administration. In several organs (lungs, ileum and nostrils), high titres of virus were evident. The E1A-deleted virus has a very restricted pattern of dissemination in cotton rats when compared to that of the wt virus. Moreover, the pattern of organ distribution was not significantly different from that observed for both routes in mice. The distribution of the E1A-deleted virus in cotton rats was close to the background of dissemination of the same virus in mice, in which the wt virus replicates poorly. Eventually, virus titres found in lungs and nasal cavities were in agreement with a lack of replication of the virus. This supports the idea that there is no significant transcomplementation of the E1A region in vivo in some types of cells. This could have been the case, as previous experiments with mouse early embryos demonstrated an E1A-like activity, also evident in ES cells (unpublished results).

Although mice have been reported to be a ‘non-permissive’ or ‘semi-permissive’ species (Prevec et al., 1989), our results show that the dissemination of both wt and dl327 viruses in mice was clearly wider than that of the E1A-deleted virus. This is evidence in favour of the capacity of the mouse species to support a low level of replication of Ad5, and indicates that results about E1A+ recombinant virus-vector vaccines in mice (Prevec et al., 1989; Wesseling et al., 1993) cannot be strictly extrapolated to that of E1A-deleted adenovirus-vector vaccines.

A major concern about the safety of E1A-deleted vaccines is the risk of transcomplementation between wt and recombinant viruses. Our results show that, even when recombinant virus is administered by a ‘safe’ route (i.m.), it can be detected in the popliteal lymph node, sometimes at high titres. Wt virus can also be isolated after i.n. inoculation (a natural route of infection) from the same lymph node, in such a way that mixing of the two populations is a possible event. It must be emphasized that in our study, a low viral dose was used in order to ensure that no deaths or clinical signs were expected in the cotton rat (Prince et al., 1993). With higher doses, it can be anticipated that more common sites of localization could have been found. Moreover, it appeared that persistence of the virus was generally not long-lasting: 1 month after inoculation, wt virus could be isolated only rarely. Nevertheless, this indicates that phenotypic complementation of E1A can occur even in people not acutely infected. We are currently conducting another set of experiments with recombinant viruses expressing either the β-galactosidase or the luciferase genes, to obtain information about the risk of excretion of such viruses in animals previously or subsequently infected with a wt virus, and to study especially the risks of phenotypic complementation or recombination.

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