Infection kinetics of human adenovirus serotype 41 in HEK 293 cells

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The purpose of this work was to acquire an overview of the infectious cycle of HAdV-41 in permissive HEK 293 cells and compare it to that observed with the prototype of the genus, Human adenovirus C HAdV-2. HEK 293 cells were infected with each virus separately and were harvested every 12 h for seven days. Infection kinetics were analysed using confocal and electronic microscopy. The results show that, when properly cultivated, HAdV-41 was not fastidious. It had a longer multiplication cycle, which resulted in the release of complete viral particles and viral stocks reached high titres. After 60 h of infection, the export of viral proteins from the infected cell to the extracellular milieu was observed, with a pattern similar to that previously described for HAdV-2 penton-base trafficking after 30 h of infection. HAdV-41 had a non-lytic cycle and the infection spread from the first infected cell to its neighbours. The release process of the viral particles is unknown. The results observed for HAdV-41 infection in HEK 293 cells show how different this virus is from the prototype HAdV-2 and provides information for the development of this vector for use in gene therapy.

Key words: Human adenovirus F - infection kinetics - virus cultivation - confocal microscopy - electronic microscopy

Human adenoviruses are members of the Adenoviridae family Mastadenovirus genus and include 51 serotypes, which are classified into six different species (from A to F) according to their biological and molecular properties. Characteristic features of each species determine cell tropism and pathogenesis, which can result in ocular, respiratory, enteric and urinary tract infections (Cusack 2005, Short et al. 2006). All adenoviruses are non-enveloped, double-stranded DNA viruses. The icosahedral virions, of approximately 90 nm in diameter, have 240 copies of trimeric hexons on the facets and a penton complex, composed of a pentameric base and a antenna-like trimeric fibre, at each of the 12 vertices. Both proteins are involved in host-cell recognition and in the initial steps of cell infection (Bergelson 1999, Coyne & Bergelson 2005).

Despite the variety of adenovirus serotypes, the data describing the virus multiplication cycle and intracellular trafficking have been obtained largely from studies of the serotypes HAdV-2 and HAdV-5, which are both members of the Human adenovirus C species. The host cell is recognised in two steps: the C-terminal globular domain of the fibre binds to the Coxsackie-Adenovirus Receptor (CAR) (Bergelson et al. 1997) and the penton-base, through its RGD (Arg-Gly-Asp) motif, subsequently interacts with the αvβ3 and αvβ5 integrins inserted in the cell membrane, resulting in the internalisation of the viral particle via receptor-mediated endocytosis (Mathias et al. 1994, Bergelson et al. 1997, Meier & Greber 2003, Coyne & Bergelson 2005). Upon acidification of the endosome, integrins and the penton-base assist in releasing the virus from the early endosome into the cytosol. The adenovirus capsid then interacts with motor proteins and is transported over microtubules toward the nucleus, where the DNA is delivered and replicated (Greber et al. 1993, 1997, Wickham et al. 1994). Newly synthesised viral proteins are carried into the nucleus and the virus progeny are assembled. Release of the virus from infected cells occurs through cell lysis.

Given the similarity in the structure of the adenovirus capsid across the genus, one might assume that the pathways of penetration, intracellular trafficking and release of any member will be representative of the group. However, this model of viral multiplication cannot be applied to all adenovirus species. Human adenovirus B uses CD46, a protein involved in complement binding, as a primary receptor (Gaggar et al. 2003). After internalisation via endocytosis, Human adenovirus B accumulates and has a long residence time inside endosomal compartments until it reaches late endosomes or lysosomes (Miyazawa et al. 2001). Release of capsids into the cytosol occurs in a lower pH environment than that of species C adenoviruses and is followed by rapid cytosolic translocation to the nucleus through an unknown mechanism (Mautner et al. 1989). Fibre protein is known to influence membrane lysis and intracellular trafficking. For example, chimeras of species C viruses carrying species B fibres show delayed intracellular trafficking (Miyazawa et al. 1999).

Serotypes 40 and 41 of Human adenovirus F (HAdV-40 and HAdV-41) have attracted considerable attention in recent years. These serotypes are important etiologi-
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**MATERIALS AND METHODS**

**Virus prototypes and cell cultures** - The cell cultures, anti-sera and adenovirus prototype strains used in this study belong to the virus and cultures collection of our laboratory and have been collected over the years from reference laboratories. The HAdV-41 Tak strain was donated by Dr. RI Glass, previously of the Centers for Disease Control, Atlanta, GA, USA. The HEK 293 cells (human embryonic primary kidney cells transformed with sheared HAdV-5 DNA) were obtained directly from Dr. FL Graham, Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada (Graham et al. 1977). The HAdV-2 prototype and HEp2 cells (human epidermoid carcinoma of the larynx) were obtained from Dr. HG Pereira, then at the Central Public Health Laboratory in London, UK. The rabbit hyperimmune serum against HAdV-5 was prepared as described previously (Peret et al. 1995).

**Cell culture** - The HEK 293 cells were cultured in a monolayer with Eagle’s Minimum Essential Medium (EMEM) supplemented with 10% of foetal bovine serum (FBS), both from Cultilab, Emcare, Campinas, Brazil. The cultures were split 1:3 every seven days, dispersed with 0.25% trypsin solution in Dulbecco’s phosphate-buffered saline, pH 7.4 (PBS) and suspended in EMEM with 10% FBS.

The HEp-2 cells were cultured in EMEM with 10% FBS, split 1:5 once a week and dispersed with 0.25% trypsin supplemented with 0.5% ethylenediaminetetraacetic acid (EDTA) in PBS. The cultures were maintained at 37°C in a 5% CO₂ atmosphere.

**Viruses strains** - The HAdV-41 strain was cultivated in HEK 293 cells. Monolayers were prepared in 75 cm² vessels (Nunc™, Thermo Fisher Scientific, Waltham, MA) seeded with 4 x 10⁶ cells. After 48 h, when the cells had reached 70% confluence, the cells were washed with PBS (pH 7.4) supplemented with 1 mM of CaCl₂ and 1 mM of MgCl₂ (PBS-A). The cells were then inoculated with a 0.1 MOI HAdV-41 stock suspension. After an adsorption period of 2 h at 37°C, the vessels were filled with EMEM supplemented with 0.2% FBS (Pieniazek et al. 1990). The inoculated cultures were observed daily until a cytopathic effect was evident (7-10 days). After three cycles of freezing and thawing, subsequent subcultures of HAdV-41 were prepared from the infected cells. HAdV-41 stock suspensions were prepared from the seventh passage in culture. Infected cells were harvested together with the culture medium and submitted to three cycles of freezing and thawing. The viral suspension was clarified by adding an equal volume of Vertrel-XF (1,1,2,3,4,4,5,5,5,5-decafluoropentane, Clarus Technology™, Hortolândia, São Paulo), followed by vigorous vortexing and centrifugation at 3,000 g for 20 min. HAdV-2 was cultivated in vessels seeded with 2 x 10⁶ HEp-2 cells. A stock suspension was prepared after the fifth passage in culture using the procedure described above.

**Virus production and purification in CsCl Gradient** - Monolayers of HEK 293 cells were prepared in twelve 185 cm² vessels (Nunc™). After two or three days, when cell culture confluence had reached 70%, the cells were washed with PBS-A and the cultures were inoculated with a 0.1 MOI seventh passage HAdV-41 stock suspension. After an adsorption period of 2 h at 37°C, each
culture was filled with EMEM supplemented with 0.2% FBS. Inoculated HEK 293 cells were incubated at 37°C in a 3% CO₂ atmosphere and observed daily. When the cytopathic effect was evident (between 7-10 days), the cells were harvested and centrifuged at 220 g for 10 min. The supernatants were then removed and the cell pellets were stored at -20°C. HAdV-2 was cultivated in twelve 185 cm² vessels seeded with HEp-2 cells. Infected cells were collected 4-5 days after inoculation.

HAdV-41 and HAdV-2 were purified in CsCl gradients prepared in 10 mM HEPES buffer at pH 7.4. Viruses were released from the infected cell pellets after three cycles of freezing and thawing. Cells were suspended in a 10 mM HEPES buffer at pH 7.4. Suspensions were clarified by adding an equal volume of Vertrel XF (Clarus Technology), followed by vigorous vortexing. Cell debris was removed by centrifugation at 2,619 g for 25 min at 4°C. Viral particles in the supernatant were purified in CsCl gradients prepared in a 10 mM HEPES buffer at pH 7.4, following a previously described protocol (Kanegae et al. 1994). Purified HAdV-41 and HAdV-2 were stored at -20°C. A portion of the purified HAdV-41 was applied to a clean side of carbon on mica grid (carbon/mica interface) and negatively stained with uranyl acetate. Micrographs were taken under low-dose conditions with a Philips CM12 transmission electron microscope (TEM, Philips, Eindhoven, Netherlands) operated at 80 kV and a nominal magnification of 15,000 times.

Polyclonal antibody production - The hyperimmune serum against HAdV-41 was prepared in a two-month-old New Zealand rabbit inoculated subcutaneously with 200 µL of purified HAdV-41 mixed with an equal volume of incomplete Freund’s adjuvant (Sigma Immunochemicals, USA). The first three inoculations were repeated at 15-day intervals and the booster was given six months later. Ten days after the booster, the animal was bled under 3% pentobarbital anaesthesia. The animal experimental protocol was previously approved by the Committee on Ethical Experimentation in Animals of the Institute of Biomedical Sciences of the University of São Paulo (116/2002).

The hyperimmune serum was separated by centrifugation and titred using the ELISA method. A 96-well microplate (TPP*, Trasadingen, Switzerland) was coated with monoclonal antibodies, diluted 1:6,400 in a carbonate-bicarbonate buffer at pH 9.5, against HAdV-41 hexon protein (Wood et al. 1989). As an antigen, 100 µL of the clarified HAdV-41 stock suspension, diluted 1:64 in PBS pH 7.4 with 0.5% Tween® 20 and 5% of dry milk (PBS/T/M), was added to each well and left for 16 h at 4°C. Serial two-fold dilutions (100 µL) of the rabbit hyperimmune serum anti-HAdV-41, prepared in PBS/T/M from 1:200-1:1024, were added to each well in duplicate. Antibodies were detected with goat anti-rabbit IgG serum labelled with horseradish peroxidase (Gibco, BRL, USA) diluted 1:6,400 in PBS/T/M. Between each step, the microplate was washed three times with PBS/T and decanted. The reaction was visualised via the addition of 100 µL substrate solution to each well (0.1 M citrate buffer, 0.2 M Na₂HPO₄, H₂O₂, and O-phenylenediamine dichloride, Sigma, USA) and read in a spectrophotometer with a 496 nm filter.

Cross-titration by indirect immunofluorescence assay (IIF) of rabbit anti-HAdV-41 and anti-HAdV-5 polyclonal sera against virus infected cells - A 96-well microplate (TPP*) was coated with HEK 293 cells (20,000 cells/well) prepared in EMEM with 10% FBS. Before inoculation, the culture medium was gently decanted and the cells were washed with PBS-A. Two-fold dilutions of the HAdV-41 stock suspension (from 1:2-1:1024) were prepared in EMEM and distributed in 10 columns of the plate, eight wells per dilution (25 µL each). The last two columns were not inoculated and were used as negative controls. After 2 h of incubation, the wells were filled with 100 µL of EMEM supplemented with 0.2% FBS. After 48 h of incubation, the cultures were washed with PBS (pH 7.4) and the cells were fixed with absolute methanol at -20°C (MERCK).

Rabbit anti-HAdV-41 serum was diluted in PBS, pH 7.2 (2-fold dilutions, from 1:80-1:2,560). Each dilution was distributed in one row of the plate: 10 wells with infected cells and two with non-infected cells (50 µL per well). The rabit pre-immune serum was diluted 1:160 and 1:320 and was distributed in the last two rows of the plate. The reaction was incubated for 1 h at 37°C. The cultures were gently washed three times with PBS, pH 7.2. Goat anti-rabbit gamma globulin (IgG) serum labelled with fluorescein (PIERCE, USA) was diluted 1:100 in PBS, pH 7.2, with Evan’s Blue (MERCK) and distributed (100 µL) to each well. The reaction was incubated for 1 h at 37°C and the cultures were washed three times with PBS, pH 7.2. To enhance fluorescence, a drop of 90% glycerol diluted in carbonate-bicarbonate buffer (pH 9.5) was added to each well and the excess was decanted. The microplate was analysed using an immunofluorescence microscope (Leica DMLS, Germany).

The same procedure of cross-titration was performed in another plate inoculated with HAdV-2 stock suspension and the rabbit anti-HAdV-5 serum. Since HAdV-2 and HAdV-5 belong to the same species and cross react, the HAdV-5 anti-serum was very good at revealing cells infected with HAdV-2. Anti-sera titres were determined as the reverse of the highest dilution where fluorescent infected cells could be unequivocally distinguished without background and where negative controls showed no fluorescence.

To remove non-specific antibodies before titration, anti-HAdV-41 and anti-HAdV-5 sera were absorbed with fresh HEK 293 cell pellets (incubated for 1 h at 37°C and centrifuged for 800 g/15 min) and HEK 293 cell protein powder extracted with acetone.

Purified virus titration (HAdV-2 and HAdV-41) by IIF - The HEK 293 cells were cultivated in a 96-well microplate (20,000 cells/well), washed with PBS-A and inoculated, in duplicate, with serial two-fold dilutions (from 1:2-1:1,024) of the purified HAdV-2 and HAdV-41 viruses. After 48 h, infected cells were washed with PBS (pH 7.4) and fixed with two drops of methanol (MERCK). Viral proteins were detected by adding anti-sera [anti-HAdV-5 (1:2,000) and anti-HAdV-41 (1:800)] diluted in PBS, pH 7.2. Infected cells were revealed with 100 µL goat anti-rabbit IgG serum labelled with fluorescein
In a 5% CO2 atmosphere. The coverslips were harvested EMEM with 0.2% FBS. Cultures were incubated at 37°C for two hours later, the wells were filled with 500 µL of MOI of one of the purified viruses diluted in EMEM. After two days, the cultures were inoculated with 0.04 µL of EMEM containing 10% FBS (50,000 cells per coverslip). Two days later, the wells were filled with 500 µL of EMEM with 0.2% FBS. Cultures were incubated at 37°C in a 5% CO2 atmosphere. The coverslips were harvested during a period of seven days at 12 h intervals. A third of the 24-well microplate with coverslips seeded with HEK 293 cells was mock infected with EMEM and used as a control. The control cultures were harvested at the same intervals. The cells were fixed with methanol at -20°C and the coverslips were stored at -20°C until the next step.

The anti-HAdV-41 and anti-HAdV-5 sera were diluted 1:800 and 1:2000, respectively, in PBS (pH 7.2), whereupon 500 µL of each dilution was added to a respective coverslip, including negative controls. The cultures were incubated for 1 h at 37°C and washed three times with PBS (pH 7.2). Subsequently, 500 µL of fluorescein-conjugated serum diluted to 1:100 in PBS (pH 7.2) with Evan’s Blue was added to each well. The cultures were incubated for 1 h at 37°C and washed three times with PBS, pH 7.2. Coverslips were mounted over microscope slides with glycerol in a carbonate-bicarbonate buffer at pH 9.5. Cells were visualised with a Zeiss LSM 510 confocal microscope (Carl Zeiss GmbH, Germany) using a pinhole setting corresponding to an optical thickness of approximately 0.5 µm. After choosing the field, cells were submitted to 543 nm and 488 nm laser scanning. Images were obtained through on-line computation and sliced into 20-25 sections. Single confocal optical slices near the middle of the infected cells were analysed with the Zeiss LSM Image Browser Version 3.2.0.115 Program (Carl Zeiss GmbH, Germany).

Epon inclusion and thin sections of infected HEK 293 cells - Monolayers of HEK 293 cells were prepared in 25 cm² vessels. After two days, when the culture confluence reached 70%, cells were washed with PBS-A and the cultures were inoculated with purified 0.04 MOI of HAdV-41. After an adsorption period of 1 h at 37°C, each culture was filled with EMEM containing 0.2% FBS and the cells were incubated at 37°C in a 5% CO2 atmosphere.

After 72 h and 156 h post-infection (p.i.), corresponding to the medium and final point of the infection kinetic, the cells were recovered and fixed with 2.5% glutaraldehyde in a 100 mM HEPES buffer (pH 7.4), post-fixed with 1% osmium tetroxide and dehydrated with ethanol. Ultra-thin sections (80 nm) of infected cells embedded in Epon were produced and collected on carbon-coated grids. The virus was produced in 293 cells, purified in a CsCl gradient and negatively stained with uranyl acetate. A: the presence of HAdV-41 on the 24-well microplate with coverslips seeded with HEK 293 cells was mock infected with EMEM and used as a control. The control cultures were harvested at the same intervals. The cells were fixed with methanol at -20°C and the coverslips were stored at -20°C until the next step.

The virus was produced in 293 cells, purified in a CsCl gradient and negatively stained with uranyl acetate. A: the presence of HAdV-41 on the 24-well microplate with coverslips seeded with HEK 293 cells was mock infected with EMEM and used as a control. The control cultures were harvested at the same intervals. The cells were fixed with methanol at -20°C and the coverslips were stored at -20°C until the next step.

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RESULTS

Purified HAdV-2 and HAdV-41 titration by IIF - HAdV-41 was efficiently propagated in HEK 293 cells and purified with a CsCl gradient. Titres obtained were 1.1 x 10⁷ FFU/mL for HAdV-41 and 2.2 x 10⁸ FFU/mL for the HAdV-2. By analysing the purified HAdV-41 with TEM, it was possible to observe a large number of complete viral particles (Fig. 1, micrograph A). The zoomed image shows long and short fibres distributed in distinct vertices of HAdV-41 (Fig. 1, micrograph B, indicated with arrows).

HAdV-41 and HAdV-2 infection kinetics assay - At 24 h p.i., a few of the cells infected with HAdV-41 presented a discrete intracellular cytoplasm fluorescence (Fig. 2A), which increased from 48 h-60 h p.i. The intensity of the cytoplasmic fluorescence was higher in cells infected with HAdV-2 (Fig. 2B). At 36 h p.i., cells infected with HAdV-2 showed cytoplasmic fluorescence and a large spread of viral proteins into the intercellular space, in a radial gradient emanating from the infected cell to neighbour cells and showing more intense fluorescence at the cell junctions (Fig. 3A-D).

At 60 h p.i., cells infected with HAdV-41 showed intense cytoplasmic fluorescence with inclusion bod-
ies (Fig. 4A, B) and a large spread of viral proteins into the intercellular space (Fig. 5A-D). Cells infected with HAdV-2, in contrast, showed nuclear fluorescence and nuclear inclusion bodies (Fig. 4C, D), as well as viral proteins in the cytoplasm. At this time point, the integrity of the HAdV-2 infected cells was still maintained.

At 72 h p.i., HAdV-41-infected cells showed nuclear fluorescence, as shown in the orthogonal projection of the image (Fig. 6A). The nuclear inclusion bodies became more evident after TEM analysis (Fig. 6, micrograph B). Cells infected with HAdV-2 were lysed and a new infectious cycle had begun all around the culture monolayer, with cells showing fluorescent cytoplasms (data not shown).

At 84 h p.i., cells infected with HAdV-41 showed granular cytoplasmic fluorescence and a discrete spreading of viral proteins or viruses to the surrounding environment (Fig. 7A). Different stages of infection were observed in cells infected with HAdV-2, including granular cytoplasmic or nuclear fluorescence, cell detachment from the coverslip surface and cell lysis (Fig. 7B).

During kinetics experiments, HEK 293 cells used as a negative control were collected every day. In Fig. 7C, a control cell collected after 84 h of incubation is shown. Cell integrity and the absence of fluorescence could be observed until 120 h. After this time, both cell monolayer retraction and cell cluster formation were observed due to the high density of cells reached after the long period of cultivation.

At 96 h p.i., a discrete spreading of viral proteins or viruses was observed in cells infected with HAdV-41. Most of the cells showed granular cytoplasmic fluorescence (data not shown). At this time, it was possible to observe an isolated cell infected with HAdV-2 undergoing lysis and presenting an extruding cytosol with the release of viral particles into the extracellular space (indicated by arrow, Fig. 8A-D).

After 132 h p.i., nuclear inclusion bodies became more evident in cells infected with HAdV-41 (Fig. 9A). The infection had spread to neighbouring cells by 144 h p.i. and at 156 h p.i. (Fig. 9B, C), it was possible to observe foci of infected cells with strong cytoplasmic or nuclear fluorescence. Retraction of the cell monolayer and the formation of clusters of infected cells detaching from the poly-D-lysine matrix were then observed. After 156 h, it was possible to observe the hexagonal arrangement of viral particles and the release of new viral progeny from the nucleus (Fig. 10, indicated by arrows).

At 168 h p.i. (Fig. 11A-D), it was possible to observe a cell with granular cytoplasmic fluorescence apparently overlapping the cell monolayer (Fig. 11D) as well as the release of viral proteins or virus particles to the nearest cells (Fig. 11B). This phenomenon was also observed in other areas of the culture with detached clusters of infected cells overlapping the culture and giving rise to new foci of infection.

Intense fluorescence was observed throughout the cell monolayer at 180 h p.i. (Fig. 11E), indicating that by then almost all of the cells were infected. Strong retraction of the cell monolayer and detachment of infected cell clusters could be observed. Even after seven days of infection, lysis was not observed in cells infected with HAdV-41.
The aim of this work was to obtain an overview of the infectious cycle of HAdV-41 and to compare that cycle to that of the Human adenovirus C prototype, HAdV-2, the best understood adenovirus. For the development of such a study, large amounts of purified viruses were required. HAdV-41 is known to grow feebly under laboratory conditions and an improved protocol for its purification therefore had to be developed. The cells were split with trypsin without EDTA. To improve virus attachment, the cell monolayers were washed with PBS-A before inoculation. Virus stocks were inoculated with a low MOI to avoid cell detachment prior to viral production. Infected cells were maintained in EMEM medium with 0.2% FBS. Viruses were recovered just before spontaneous cell detachment (between 5-7 days). Finally, viruses were extracted from large amounts of infected cells and CsCl purification was preceded by clarification with Vertrel-XF (Clarus Technology®) to promote the removal of membranes. Titres of the recovered HAdV-41 infectious particles were approximately 20 times lower than that of HAdV-2. Although this seems to be a low ratio, it is higher than those obtained with other methods previously used to produce HAdV-41 (Brown 1985). In a recent study using HAdV-41 as a vaccine vector, the efficient rescue of infectious particles, obtained after transfection of HEK 293-ORF6 cells with purified DNA, was 100 times lower than that of the HAdV-5 vector (Lemiale et al. 2007).

**DISCUSSION**

There are three good reasons for studying the species F human adenoviruses (HAdV-40 and HAdV-41). First, they are important etiological agents of infantile gastroenteritis (de Jong et al. 1983, Uhnoo et al. 1984, Kidd et al. 1986). Second, adenoviruses may take advantage of an impaired or destroyed immune system to set up persistent or generalised infections in an immune-compromised host (Hierholzer 1992). Third, they have been proposed as gene transfer vectors for intestinal epithelial cells (Croyle et al. 1998) and as vaccine vectors (Lemiale et al. 2007).

The aim of this work was to obtain an overview of the infectious cycle of HAdV-41 and to compare that cycle to that of the Human adenovirus C prototype, HAdV-2, the best understood adenovirus. For the development of such a study, large amounts of purified viruses were required. HAdV-41 is known to grow feebly under laboratory conditions and an improved protocol for its purification therefore had to be developed. The cells were split with trypsin without EDTA. To improve virus attachment, the cell monolayers were washed with PBS-A before inoculation. Virus stocks were inoculated with a low MOI to avoid cell detachment prior to viral production. Infected cells were maintained in EMEM medium with 0.2% FBS. Viruses were recovered just before spontaneous cell detachment (between 5-7 days). Finally, viruses were extracted from large amounts of infected cells and CsCl purification was preceded by clarification with Vertrel-XF (Clarus Technology®) to promote the removal of membranes. Titres of the recovered HAdV-41 infectious particles were approximately 20 times lower than that of HAdV-2. Although this seems to be a low ratio, it is higher than those obtained with other methods previously used to produce HAdV-41 (Brown 1985). In a recent study using HAdV-41 as a vaccine vector, the efficient rescue of infectious particles, obtained after transfection of HEK 293-ORF6 cells with purified DNA, was 100 times lower than that of the HAdV-5 vector (Lemiale et al. 2007).
results show that it is possible to obtain good titres of infectious HAdV-41, with the recovery of complete viral particles (as shown by electron microscopy), when the culture conditions described above are used and enough time is given to complete the virus multiplication cycle.

A polyclonal rabbit anti-HAdV-5 antibody produced in the laboratory for another study (Peret et al. 1995) proved to be very useful for revealing HAdV-2 proteins in infected cells. These two serotypes belong to the same species and are known to cross-react in serology. In contrast, when this serum was applied to cells infected with HAdV-41, a non-satisfactory result was obtained. Therefore, we prepared a polyclonal serum in a rabbit inoculated with purified HAdV-41. The serum obtained gave good titres and was successfully used in the kinetics study. The monoclonal antibodies to HAdV-41 (hexon protein) worked well when titrating the virus by ELISA, but gave poor results in immunofluorescence studies.

For the kinetics assays, a low MOI (0.04) was used for both viruses. The main reason for choosing such a low MOI was the fact that one of the receptors used by adenoviruses, CAR, is a cell adhesion molecule and a component of the tight-junctions. At the beginning of the viral multiplication cycle, a large amount of pentons (bases and fibres) is released from the infected cells (Walters et al. 2002). It is known that in infections with a high MOI of adenoviruses, the excess of pentons causes a cytotoxic effect with rapid destruction of the cell monolayer. As a consequence, analyses of the virus infection kinetics would not be possible. Cells infected with a low MOI made it possible to carefully observe the spread of the infection to neighbouring cells.

The HEK 293 cells were infected with the same MOI of HAdV-41 and HAdV-2. At 24 h, cultures infected with HAdV-41 showed a few cells with a granular fluorescent cytoplasm and discrete intercellular fluorescence. The fluorescence increased in intensity through the 48 h time point. At 60 h, infected cells presented intense cytoplasmic fluorescence with inclusion bodies and a large spread of viral protein in the intercellular space, in a radial gradient emanating from the infected cell to neighbouring cells. This process was delayed 24-30 h relative to that of HAdV-2. The spreading of HAdV-41 viral proteins into the extracellular milieu, visualised with a polyclonal anti-serum, showed the same pattern as observed by Trotman et al. (2003) for an HAdV-2 infection in A549 cells (human lung carcinoma), visualised with monoclonal antibodies to penton-base. Although there are known differences in the HAdV-41 penton-base and fibre sequences (Kidd et al. 1990), likely resulting in the
receivng of different receptors (Albinsson & Kidd 1999), these proteins were shown to be exported in the same manner as HAdV-2 pentons, possibly through a Golgi-independent mechanism (Trotman et al. 2003).

At the beginning of the infection cycle, before the assembly of viral progeny (from 30-60 h), an excess of penton-bases and fibres are released from the infected cells and these proteins interact with receptors such as CAR, integrins and also with heparan-sulfate in the intercellular space. Early release of penton-bases and fibres will dissociate cell junctions and possibly prepare surrounding cells for infection (Walters et al. 2002). This might be an important strategy of this virus to make permeable the gut columnar epithelium and facilitate viral spreading.

The HAdV-41 multiplication cycle had a slower progression than that of HAdV-2. Newly produced viral particles accumulated inside the cell nucleus from 72-132 h p.i. Viral progeny were then released from the infected cells into neighbouring cells, showing a focal dispersion of the infection. To observe whether newly produced viruses pass from cell to cell or are released into the extracellular space, infectivity experiments were repeated with the addition of a neutralising serum to the culture medium at 72 h p.i. (data not shown). These antibodies partially reduced viral spreading, suggesting that HAdV-41 progeny are liberated from the host cell through a non-lytic and likely non-Golgi-dependent, unknown mechanism. In contrast, HAdV-2 caused lysis, with subsequent infection of the whole cell culture, as previously described (Tollefson et al. 1996b, 2003). The efficient release of HAdV-2 virus in the lytic cycle occurs due to the adenovirus death protein (ADP). This protein is exclusively present in Human adenovirus C and is expressed by the 11.6K ORF of the E3 region (Tollefson et al. 1996a, b). All of the other human adenovirus species lack this ORF. HAdV-41 does not express ADP and consequently does not lyse infected cells. In this study, it was observed that with the progression of the HAdV-41 infection, groups of cells retracted, lost adhesion and detached from the ma-


