Washout Kinetics of Viral Vectors from Cultured Mammalian Cells

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Abstract

The handling of infectious viral vectors such as adenovirus type 5 (Ad5), lentivirus (HIV1), and vaccinia virus (VV) requires biosafety level 2 (BSL-2) containment. This also applies to modified vaccinia Ankara (MVA) and adeno-associated virus type 2 (AAV2) provided that the insert involves additional hazard. To transfer infected cells to the lower BSL-1 containment, viral particles have to be demonstrably cleared from the supernatant as specified by biosafety guidelines. With the objective to provide data about the washout kinetics using common culture techniques, the authors infected different adherent mammalian cells with the aforementioned viral vectors. The supernatant of the cell culture was subsequently monitored for the presence of viral nucleic acids during various steps of washing and cell passaging. Complete clearance could be demonstrated for Ad5 when infecting Hela cells and for lentivirus (HIV1) in HEK293T cells. No or an undefined clearance was detected for AAV2, VV, and MVA in Vero-B4 cells and Ad5 after infection of HEK293 cells. Results demonstrated that virus persistence in the supernatant was greatly influenced by the washing procedure, the number of passages, and the vector titer, as well as the type of host cell line. The authors therefore conclude that procedures for clearance cannot be predefined for given virus-host systems. An analysis of the supernatant should be performed for each individual experimental setup prior to downgrading the risk class and subsequently the containment level used.

Introduction

Adenovirus type 5 (Ad5), HIV1-derived lentivirus, adeno-associated virus (AAV), and vaccinia viruses are well established tools for gene transfer *in vitro* and for gene therapy (Bouard et al., 2009; Howarth et al., 2010). These vectors exhibit distinct characteristics concerning transgene delivery and expression as well as replication capacity and biosafety. Ad5 and lentiviral (HIV1) vectors have been generated by removing parts of the original viral genome. For replication, they depend on specific host cells or the addition of helper plasmids (Palmer & Ng, 2005; Pauwels et al., 2009). Supranational guidelines for contained use of these viral vectors agree that they be handled at biosafety (containment) level 2 (BSL-2) (Eidgenössische Fachkommission für biologische Sicherheit (EFBS), 2009; Health and Safety Executive (HSE), 2007; National Institute of Health (NIH), 2011; Zentrale Kommission für Biologische Sicherheit (ZKBS) 2001, 2007). In contrast, vaccinia virus (VV) is used in wild-type form. VV is the vector of choice for some applications where high expression rates are needed and for immunization (MacNeil et al., 2009). Work with VV has to be conducted at BSL-2 (Eidgenössische Fachkommission für biologische Sicherheit (EFBS), 2009; Health and Safety Executive (HSE), 2007; National Institute of Health (NIH), 2011; Zentrale Kommission für Biologische Sicherheit (ZKBS), 1997). Modified Vaccinia Ankara (MVA), which has been generated by serial passage in chicken embryo fibroblasts (CEF), exhibits a highly reduced replication capacity (Meyer et al., 1991). Thus, BSL-2 handling is compulsory only when the transgene implicates a supplementary risk (Eidgenössische Fachkommission für biologische Sicherheit [EFBS], 2009; Health and Safety Executive [HSE], 2007; National Institute of Health (NIH), 2011; Zentrale Kommission für Biologische Sicherheit [ZKBS], 2002). Adeno-associated viruses (AAV) are generally regarded as non-pathogenic because replication and release of viral particles take place only during superinfection with a helper virus (adenovirus, herpes simplex virus [Berns & Giraud, 1996]). The most commonly used serotypes of AAV (AAV2, 3, 5) have been classified as risk (hazard) group 1 (RG1), unless helper viruses are employed (Eidgenössische Fachkommission für biologische Sicherheit (EFBS), 2009; Health and Safety Executive (HSE), 2007; National Institute of Health (NIH), 2011; Zentrale Kommission für Biologische Sicherheit (ZKBS), 2005). However, evidence for insertional mutagenesis at the preferred integration site AAVS1 in the host genome, as well as the fact that cellular genotoxic stress can induce a lytic response, has led to considerations about a reassessment of AAV (Donsante et al., 2007; Lambert, 2008; Yalkinoglu et al., 1988).

By biosafety regulation, derogation of virally infected cells from RG2 to RG1, and thus their contained use at BSL-1 instead of BSL-2, is possible given that no viral particles are detectable in the supernatant of the infected cells. Since the user prefers to carry out experimental work post infection at BSL-1, the time and the procedure required to clear all viruses from the cell culture supernatant are desirably minimal. As an official biosafety laboratory, the authors support the federal authorities in Switzerland in issues related to the Swiss Ordinance on the Contained Use of Organisms (The Federal Authorities of the Swiss Confederation, 1999). They analyzed procedures for clearing viral particles from cell culture for individual strains of Ad5, lentivirus (HIV1), VV, MVA, and AAV2. Several types of adherent mammalian cells were infected with these viral vectors and the supernatant of the cell culture was monitored for the presence of viral nucleic acids after infection and subsequent cell passage. As a conclusion, a general statement of the derogation of the containment level is made.

Materials and Methods

Plasmids, Viral Specimens, and Cell Lines

Adenovirus Type 5 (Ad5) construct Ad5ΔE1GFP (E1 region of Ad5 replaced by a green fluorescent protein expression cassette; replication-deficient) (Ehrengruber et al., 2000; Qu et al., 1998) was a kind gift from Markus Ehrengruber, Kantonsschule Hohe Promenade, Department of Biology, Zürich, Switzerland. The pLL3.7 plasmid (lentiviral vector) (Rubinson et al., 2003) as well as the helper plasmids pMD2.G, pRSV-Rev, and pMDLg/pRRE (Dull et al., 1998) were obtained from Addgene Inc. (Cambridge, MA, USA). Lentivirus (HIV1) pLL3.7 stock solutions were prepared as described earlier (Bagutti et al., 2011) and concentrated by Lenti-X™ Concentrator (Clontech, Takara Bio Europe, St. Germain-en-Laye, France) according to the manufacturer's protocol. Adeno-associated virus serotype 2 (AAV2; ATCC-VR-680, [Hoggan et al., 1966]) was purchased from LGC Standards (Teddington, UK). Vaccinia virus VVeGFP Western Reserve was kindly provided by Maries van den Broek, Department of Oncology of the University Hospital Zurich, Switzerland. The EGFP gene has been inserted into the Western Reserve (WR) wild-type strain as described by Moss & Earl (1998). Modified vaccinia Ankara (MVA, ATCC-VR-1508, [Antoine et al., 1998]) was purchased from LGC Standards. HEK293 (ACC 305), HEK293T (ACC 635), Hela (ACC 57), and Vero-B4 (ACC 33) cells were obtained from DSMZ (Braunschweig, Germany).

Cell Culture

Cells were routinely cultivated in standard cell culture flasks (TPP, VWR, Dietikon, Switzerland) at 37°C with 6% CO_2 using standard culture medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Sigma-Aldrich, Buchs, Switzerland) as well as either 27 µg/ml Geneticin® (G418; Gibco, Invitrogen, Basel, Switzerland) for Vero-B4 or L-Glutamine (200 µM), Penicillin (10 units/ml), and Streptomycin (10 µg/ml) (Sigma) for HEK293, HEK293T, and Hela. Routine procedures were performed as follows: The cells were washed by adding and subsequently removing 1x PBS (Sigma); repeated once. Passaging of the cells was performed by washing (as described above) followed by the addition of 1x trypsin/EDTA (Sigma) for approximately 2 minutes before stopping the reaction and removing the cells by adding cell culture medium (in a five-fold excess to trypsin). The cells were reseeded directly in 1/5 to 1/20 dilutions depending on the cell density.

Viral Infections

Ad5 infection of HEK293 and Hela cells was performed by adding Ad5ΔE1GFP stock solution (two separate viral preparations) to wells of a 12-well cell culture plate (TPP, VWR, Dietikon, Switzerland) before the cells were added, leading to a confluency of 70% (Bagutti et al., 2011). Twenty-four hours later the medium was exchanged (standard protocol) and the cells were incubated for another 3 days. Where indicated, additional washing steps (twice with PBS at room temperature or twice with standard supplemented DMEM at 37ºC for 5 minutes each) were carried out prior to medium exchange. The cells were then passaged routinely up to 14 days or until cell death occurred. For lentivirus (HIV1) infection of HEK293T, the cells were seeded in advance in poly-L-lysine-coated 6-well plates (BD Biocoat™ [Becton Dickinson, Franklin Lakes, NJ], VWR, Switzerland) to reach a confluency of 70% the day of infection. One hour prior to the addition of pLL3.7 lentiviral stock solution (two separate viral preparations), the cells were treated with 8 µg/ml Polybrene. Five hours post infection, the cells were washed twice rigorously with 1x PBS and incubated for another 3 days. The cells were then passaged routinely as indicated for up to 15 days. For AAV2 infection, HEK293 and Hela cells were seeded the previous day in 6-well plates to reach a confluency of 70% on the day of infection. The medium was removed except for 500 µl before 10 µl of AAV2 stock solution (ca. 10^4 and 10^6 genome copies/ml) were added and incubated in the presence or absence of 3 µM etoposide (Sigma-Aldrich, Buchs, Switzerland). After 2 hours, the medium was replenished to 3 ml. The cells were then passaged routinely as indicated until cell death occurred or for up to 14 days. VVeGFP and MVA infection of Vero-B4 was performed by adding VVeGFP or MVA, respectively, and basically according to the same protocol as for AAV2 (with the deviation that the medium did not contain any etoposide and the medium was replenished 1 hour post infection to 1.5 ml). The cells were passaged routinely as indicated for up to 8 days and 35 days, respectively, or until cell death occurred.

Sampling and DNA/RNA Extraction

To extract nucleic acids, 200 µl of supernatant or 200 µl cell suspension were used. Cell suspensions were generated by detaching the cells from one well as described for routine passaging and resuspending the pellet in 200 µl 1x PBS. For the detection of Ad5, AAV, and vaccinia virus, DNA was extracted according to the QIAamp DNA Mini Kit protocol (Qiagen, Basel, Switzerland) in combination with the extraction robot QIAcube (Qiagen) according to the manufacturer's standard procedure. Lentiviral (HIV1)-specific nucleic acids (DNA and RNA) was extracted using the QIAamp Viral RNA Mini Kit (Qiagen) as described earlier (Bagutti et al., 2011).

Detection of Virus-Specific Genes and Human RNase P Control Gene in Nucleic Acid Extracts by Real-Time PCR

The following primers and probes were used: for the detection of Ad5: Ad5fiber-F (5'-aag cta gcc ctg caa aca tca-3', at a final concentration of 100 nM), Ad5fiber-R (5'-ccc aag cta cca gtg gca gta-3', 300 nM), and Ad5fiber-FAM (FAM-5'-cct cac cac cac cga tag cag tac cct tac-3'-TAMRA, 100 nM) (Bagutti et al., 2011); for lentivirus (HIV1): HIV-1-PSS-F (5'-cgc agg act cgg ctt gct-3', 400

nM), HIV-1-PSS-R (5'-gac gct ctc gca ccc at-3', 400 nM) and HIV-1-PSS-Pr (5'- FAM-ccy ctc gcc tct tgc ygt gyg crc -TAMRA-3', 150 nM) (Bagutti et al., 2011); for AAV2: AAV-WT-F (5'-agt tga act ttg gtc tct gcg tat t-3', 500nM), AAV-ITR3-R2 (5'-cat cac tag ggg ttc ctt gta gtt aat-3', 500nM), and AAV-ITR3-VIC (VIC-5'-att aac ccg cca tgc tac tta tct acg tag cc-3'-TAMRA) for the detection of a sequence covering parts of the Rep protein genes and the inverted terminal repeats (ITR) of AAV2 (GenBank® Acc.Nr. AF043303: 4446-4525); for VVeGFP: VvI4L-F (5'-gac act ctg gca gcc gaa at-3', 500nm); VvI4L-R (5'-ctg gcg gct aga atg gca ta-3', 500 nm), and VvI4L-VIC (VIC-5'-agc agc cac ttg tac tac aca aca tcc gga-3'-TAMRA, 150 nm) for the detection of a fragment of the ribonucleotide reductase I4L (GenBank® Acc.Nr. M35027: 67'133-67'195); for MVA: VvTK-F (5'-tcg atg aag gac agt tct ttc ca-3', 600 nM), VvTK-R (5'-cca tcg agt gcg gct act ata a-3', 200 nM) and VvTK-FAM (FAM-5'-ttg cca tac gct cac aga att caa caa tgt-3'-TAMRA, 200 nm) for the detection of a fragment of the thymidine kinase gene of the vaccinia virus MVA (GenBank® Acc.Nr. M35027: 84'096-84'186). AAV, VvI4L, and VvTK primers and probes were designed in-house using Primer Express® Software (Applied Biosystems, Rotkreuz, Switzerland) and tested *in silico* for specificity and cross-reactivity using BLAST from the National Center for Biotechnology Information (Altschul et al., 1990). All DNA/RNA samples were amplified in 25 µl reactions containing 5 µl template DNA/RNA. Lentiviral (HIV-1) HIV-1-Ψ copies (DNA) and genomes (RNA) and Ad5 fiber gene copies were detected and quantified as described earlier (Bagutti et al., 2011). Real-time PCR for the detection of Ad5, AAV2, VV, and MVA gene copies were performed on the StepOne™ Real-time PCR System (Applied Biosystems) using 1x TaqMan® Fast Universal PCR Master Mix (Applied Biosystems) with the following parameters: initial heating to 95ºC for 20 seconds followed by 45 cycles of 95ºC for 3 seconds and 60ºC for 30 seconds. Quantification of VV and MVA DNA gene copies were performed by serial dilutions of reference plasmids p-VV-I4L and p-VV-TK, respectively. AAV2-specific genome copy numbers were calculated by approximation using serial dilutions of AAV2 stock solution and by applying a reference line with assumed ideal quantitative PCR conditions (i.e., Ct 20 equals approximately 10^6 gene copies). AAV2-specific gene copies are not interpreted as stand-alone results but as comparisons referred to the initial concentration.

Production of Reference Plasmids for the Real-Time PCR of VV and MVA

The reference plasmids for VV and MVA were produced by subcloning a 225-bp fragment containing the target region of the I4L gene (M35027: 67'066-67'290) and a 250-bp fragment containing the target region of the thymidine kinase TK gene (M35027: 84'017-84'267), respectively, into the pGEM-T vector system (Promega, Dübendorf, Switzerland).

Results and Discussion

Adenovirus Type 5 (Ad5)

Infection in Hela cells with Ad5ΔE1GFP (5 x 10^4, 2 x 10^5 and 2 x 10^6 pfu/ml) was confirmed by detection of the Ad5 fiber gene by real-time PCR after extraction of DNA from the cell monolayer (data not shown). To monitor the clearance from the supernatant, samples were taken 5 hours (PI0) and 24 hours post infection (PI1) before washing. Thereafter, sampling of the supernatant took place on PI4, PI5, PI7, PI8, PI11, PI12, PI14, PI15, and cells were passaged routinely up to four times (Figure 1a). As judged by the presence of Ad5 fiber DNA sequences in the supernatant of Hela cells, the decrease of Ad5 until the cells started to detach (depending on the experiment after PI1, PI4, PI5, or PI7; Figure 1a). Less than 3% of the initial Ad5 genome copies number (PIO) could be detected on PI4. From PI5 onwards, including one cell passage, Ad5 DNA was detectable only in two instances (Figure 1a). As Ad5 mediates transient gene expression, Ad5-infected cells are often used for further experimentation before being passaged. Therefore, the study analyzed the process of washing on PI1 in more detail. Three different washing protocols prior to the addition of fresh medium were compared: untreated, routine washing with PBS twice for a few seconds at room temperature or washing with medium twice for 5 minutes at 37ºC each. Ad5 genome copies declined significantly from PI0 to PI3 (in absolute numbers approximately 10^7 genome copies/ml vs 10^6 and 10^5 for the respective titer, as well as judged by the ratio of Ad5-specific gene copies/ml of PI3 vs PI0; Figure 1b). Whereas the degree of the decline was dependent on the titer used for infection, it was not influenced by the washing protocol. Ad5ΔE1GFP did not produce viral particles in Hela cells and clearance, therefore, could not be observed. However, the time and the degree of clearance were strongly influenced by the Ad5 titer applied. Consequently, it has to be investigated for each experimental setup of Ad5 infection before derogating the risk class. As proof of principle, Ad5ΔE1GFP (5 x 10^4 and 2 x 10^5 pfu/ml) was used to infect HEK293 cells, which are able to complement the deleted E1 genes of the replication-deficient Ad5ΔE1GFP, resulting in a steady increase of Ad5 fiber gene copies in the cell supernatant (Figure 1a). New viral particles were constantly produced until all cells were infected and eventually lysed after PI6. Thus, clearance could not be achieved.

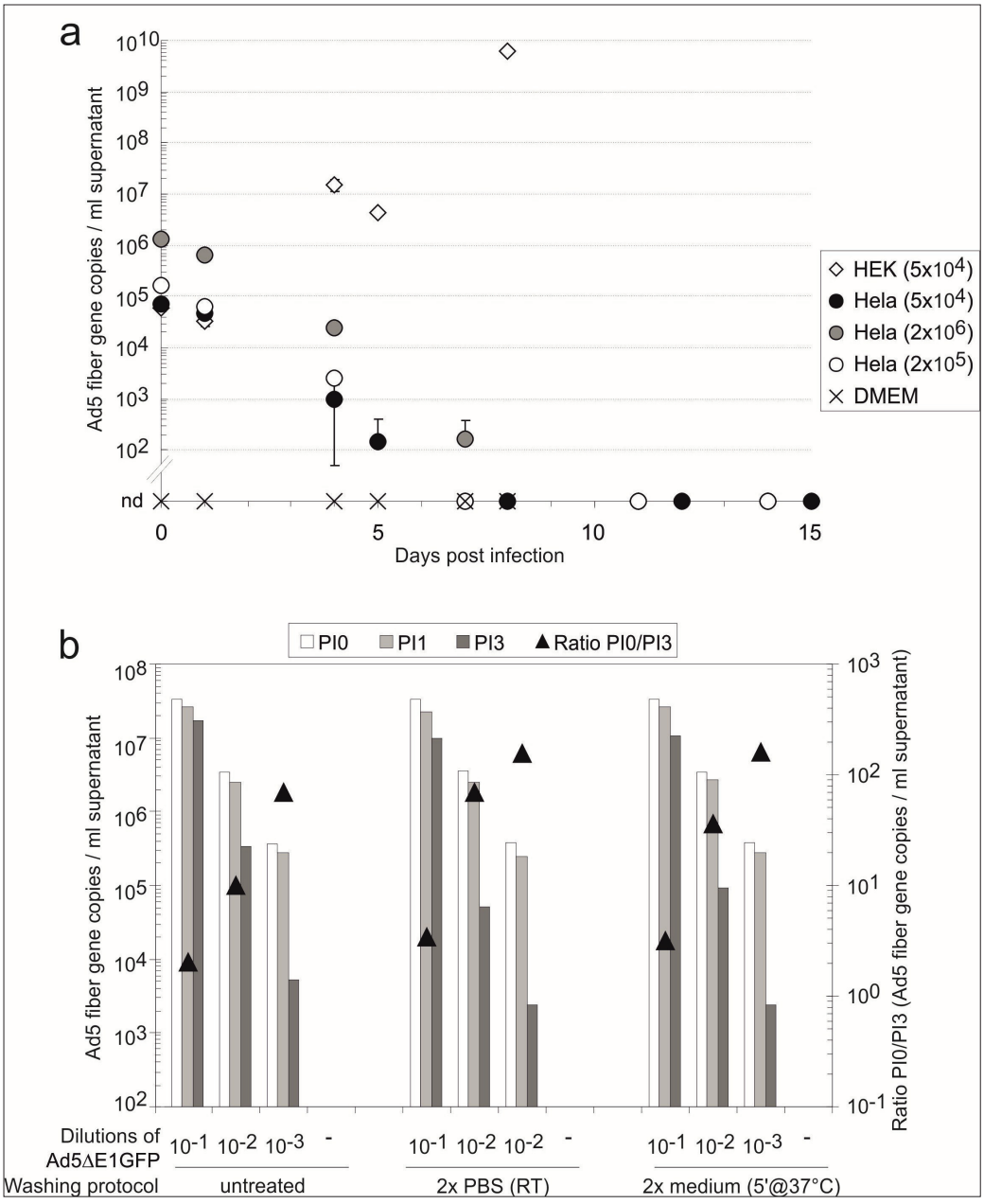
Lentivirus (HIV1)

HEK293T cells were infected with pLL3.7 lentiviral (HIV1) vector (at three different titers 10^3, 3 x 10^3, and 3 x 10^4 TU/ml). This construct does not contain any structural or replication-relevant genes; thus, it is incapable of replication. Successful infection and integration of the transgene into the host genome were monitored by screening the cell monolayer microscopically for the expression of GFP (Figure 2a). Five hours post infection (PI0) and prior to the washing step and medium substitution, control samples

were taken from the cell supernatant. During the following 15 days, the cell supernatant was routinely sampled before passaging (PI1, PI4, PI7, PI11) and 1 day after passaging (PI5, PI8, PI12) as well as thereafter on days PI14 and PI15. The virus titer in the supernatant was determined by RNA extraction and subsequent detection of HIV-Ψ RNA gene copies by real-time RT-PCR. One day and two rigorous washing steps after infection, the number of lentivirus (HIV1)-specific RNA gene copies was significantly reduced by approximately 100-fold (Figure 2b). After having passaged the cells once, the HIV1-Ψ-RNA copies fell ≤ 1% of the initial amount. Although the initial concentration varied between 3 x 10^5 and 5 x 10^7 genome copies/ml, clearance was achieved by two cell passages. The constant

Figure 1

Clearance of Ad5: Ad5ΔE1GFP were used to infect HEK293 (a) and Hela (a, b) cells. The supernatant was analyzed for the presence of Ad5-fiber gene copies by real-time PCR at different time points post infection as indicated (days post infection, PI). The results are presented as mean ± SD. **(a)** Ad5ΔE1GFP titers used: 5 x 10^4 pfu/ml (HEK:, 2 Hela: 3 experiments), 2 x 10^5 (Hela: 2 experiments) and 2 x 10^6 (Hela: 2 experiments). Washing (PI1) and passaging steps (PI4, PI7, PI11, PI14) were performed immediately after sampling. **(b)** Ad5ΔE1GFP titers used: 10^{-1}, 10^{-2}, 10^{-3} dilutions of 2 x 10^6 pfu/ml. On PI1 (after sampling) different washing procedures or no treatment (as indicated) were performed. Secondary axis on the right shows ratios of genome copies on PI0 to genome copies on PI4. Medium control (-). Limit of detection (LOD, 200 gene copies/ml); nd: not detected.



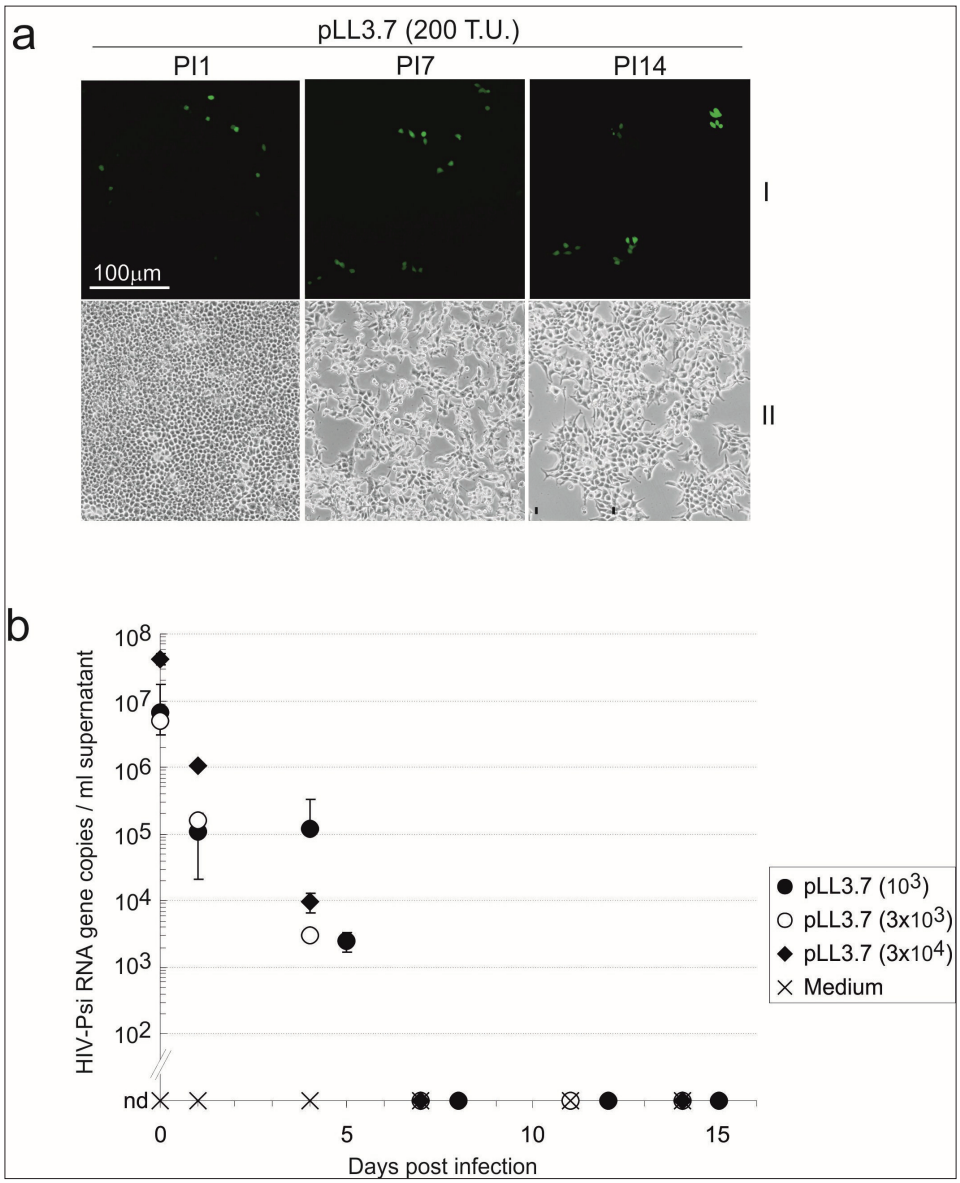
expression of viral GFP at least until PI14 in parallel without secretion of viral particles demonstrated stable integration of viral genome in the host cell genome (Figure 2a vs 2b). Derogation of the containment level of lentivirus (HIV1)-infected HEK293T is therefore practicable.

Adeno-Associated Virus Serotype 2 (AAV2)

Two different titers of wild-type AAV2 (ca. 10^4 and 10^6 genome copies/ml) were used to infect Hela cells (Figure 3). Two hours after infection of Hela cells, the medium was replenished and control samples were taken from the supernatant. As AAV2 did not express a marker protein visible by microscope, successful AAV2 infection was detected by analyzing AAV-ITR DNA copies in the host cells compared to those in the supernatant on PI1, PI6, and PI9 by real-time PCR (Figure 3a). To improve infection efficiency, etoposide was used in a second approach. The incorporation of AAV2-specific DNA sequences was confirmed and an increase of the ratio of AAV2 genome copies in the cell pellet to the supernatant was observed by up to 10^4-fold on days PI6 and PI9. To monitor the clearance rate of viral genome in the supernatant, samples were taken on PI1 and thereafter on days PI4, PI7, PI11, and PI14 in each case before cell passage. AAV2-specific DNA sequences

Figure 2

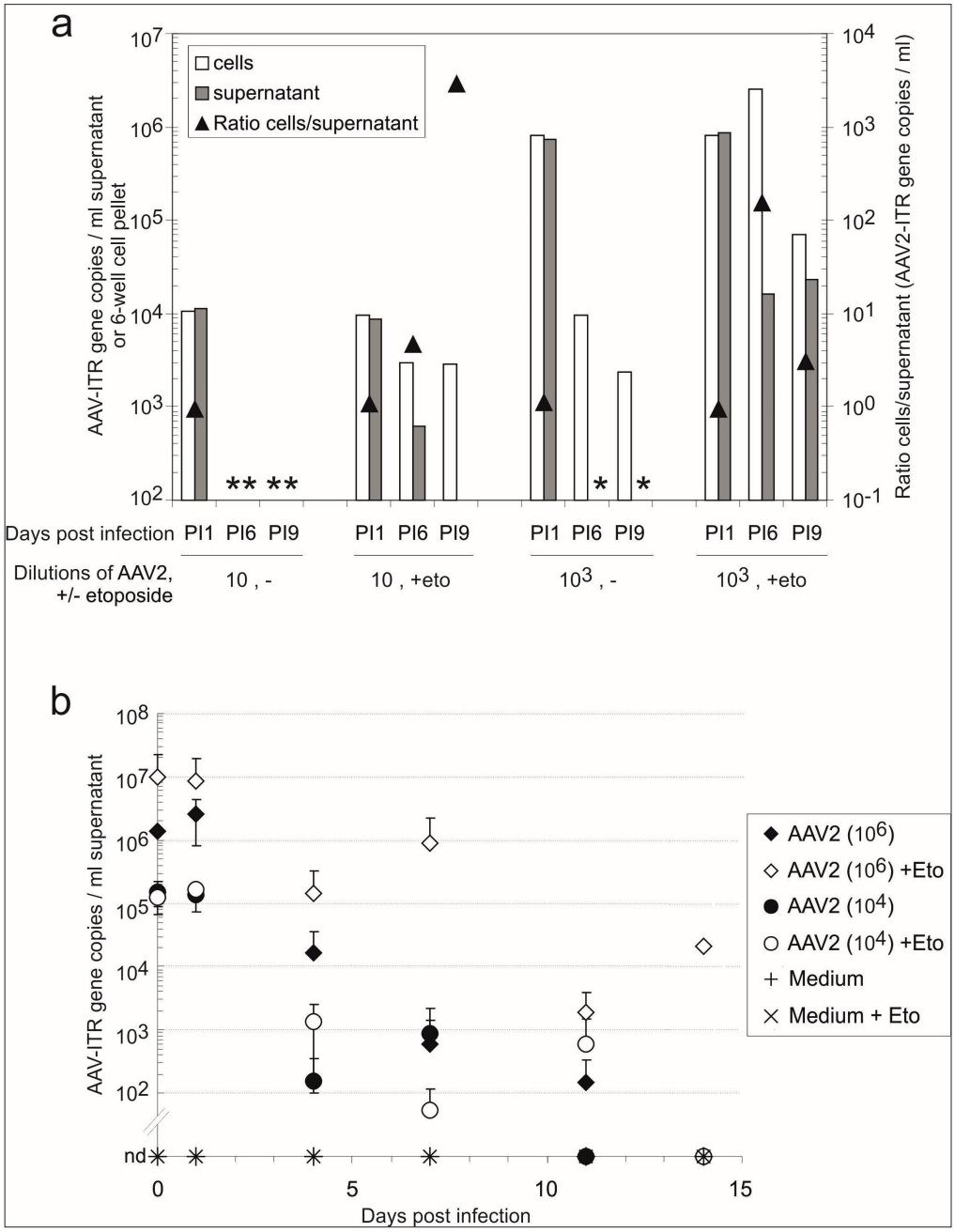
Clearance of lentivirus (HIV1): HEK293T cells were infected with pLL3.7 lentivirus (HIV1) (10^3, 3 x 10^3 and 3 x 10^4 TU/ml). **(a)** Incorporation of GFP was analysed by fluorescence microscopy 24 hours (day 1) post infection (PI1), 7 (PI7) and 14 days (PI14) post infection (panel I). As a control, the respective phase contrast is shown (panel II). **(b)** The supernatant was analysed for the presence of HIV-Ψ-gene copies by real-time PCR at different time points post infection as indicated. Washing (PI0) and passaging steps (PI4, PI7, PI11) were performed immediately after sampling. The graph shows the results of 2 individual experiments per titer. Limit of detection (LOD, 257 gene copies/ml); nd: not detected.



were still detectable in the supernatant even after four rounds of passaging (Figure 3b). Particularly, etoposide-assisted infections caused higher numbers and a longer persistence of AAV2 genome copies in the supernatant. AAV2 is only conditionally replicative. Several factors including cellular stress and superinfection can induce the release of viral particles (Berns & Giraud, 1996; Yalkinoglu et al., 1988). Since at least in the absence of etoposide no such factor was added, the authors' finding contrasted with the replication-deficient characteristics of AAV2. Furthermore, Meyers et al. (2009) has reported rescue of AAV2 from differentiated keratinocyte in the absence of helper viruses or genotoxic agents. The cause of the persistence of AAV nucleic acids in the supernatant of the Hela

Figure 3

Clearance of AAV2: AAV2 was used to infect Hela cells in the presence or absence of etoposide as indicated. The supernatant (a, b) and cell pellet (a) were analyzed for the presence of AAV-ITR gene copies by real-time PCR at indicated time points post infection (days post infection, PI). **(a)** AAV2 titer used: 10 and 10^3 genome copies/ml, respectively. Secondary axis on the rights indicates ratio of genome copies integrated vs supernatant. One and two asterisks (*/**), respectively, indicate that gene copies were not detected in one or both duplicates. **(b)** AAV2 titer used: ca. 10^4 and 10^6 genome copies/ml, respectively, summarizing 2 experiments per titer. Passaging steps (PI1, PI4, PI7, PI11) were performed immediately after sampling.. Limit of detection (LOD, 200 gene copies/ml); nd: not detected; eto: etoposide.



cells was not entirely clear in this setup and no distinct number of passages can be given for complete clearance.

Vaccinia Virus (VV) and Modified Vaccinia Ankara (MVA)

Infection of replication-competent VV leads to replication of the virus in the cytoplasm of host cells and subsequent release into the supernatant. This could be confirmed by infecting Vero-B4 cells with VVeGFP (4 x 10^4 pfu/ml) and monitoring the supernatant for the presence of I4L gene copies by real-time PCR 5 hours post infection (PI0) as well as on PI1, PI4, PI5, and PI8 (Figure 4a). Over this period the cells were routinely passaged twice before they started to come off. An increase of VV-specific DNA in the

Figure 4

Clearance of VV and MVA: Vero-B4 cells were infected with **(a)** VVeGFP (4 x 10^4 pfu/ml, 2 experiments) and **(b)** MVA (2 x 10^4 and 2 x 10^6 pfu/ml, respectively, summarizing 4 experiments each titer). The supernatant was analysed for the presence of vaccinia I4L- **(a)** and TK4 **(b)** gene copies, respectively, by real-time PCR at different time points post infection as indicated (days post infection, PI). Passaging steps (PI1, PI4, PI7, PI11, PI14, PI18, PI21, PI25, PI28, PI32) were performed immediately after sampling. Limit of detection: 200 gene copies/ml; nd: not detected.

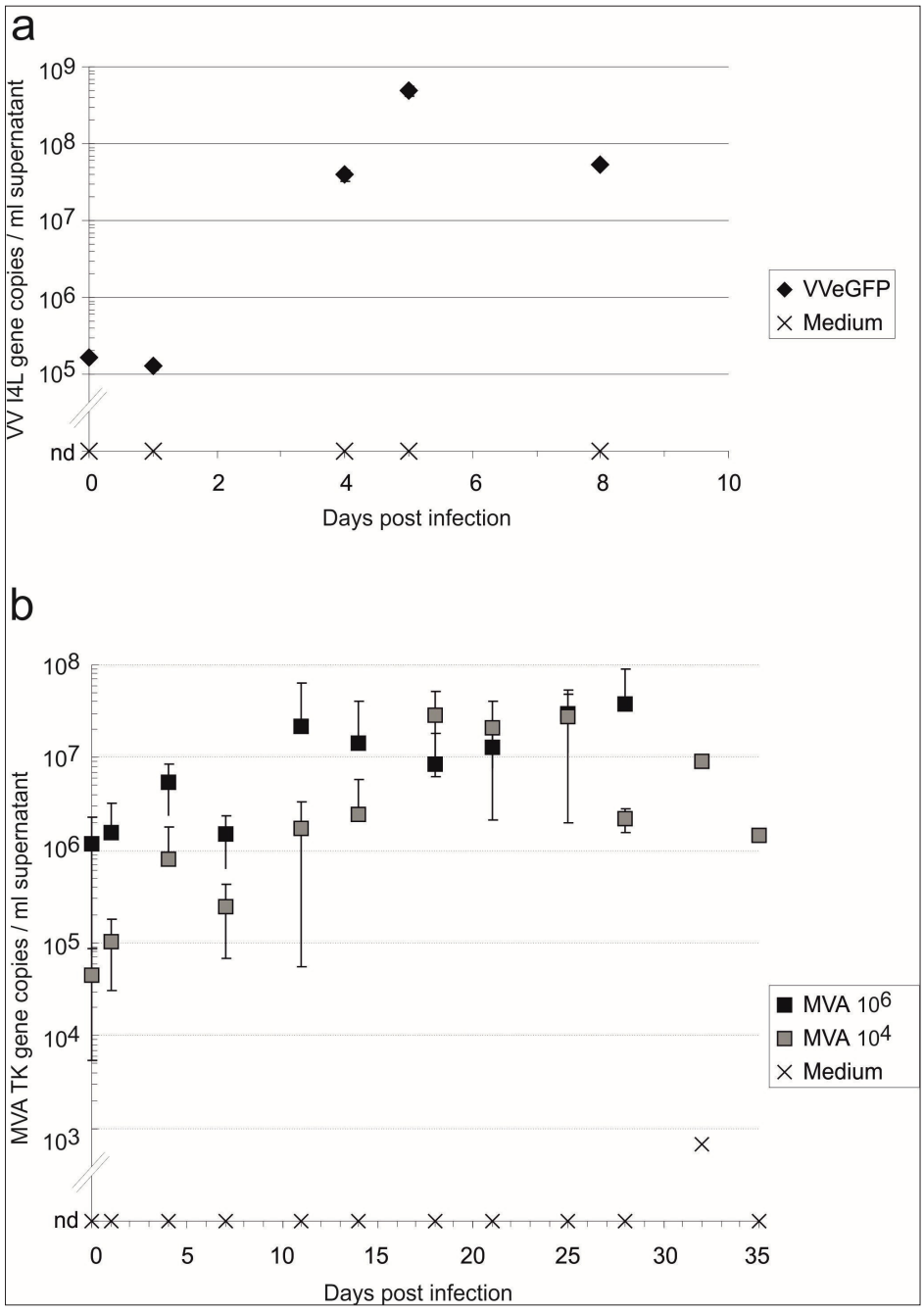


Table 1

Summary of the results and recommendation for downgrading the biosafety level.

Virus (strain/serotype)	Replication Capacity	Host Cells Used	Washing Out (no. cell passages[§]; remark)	Down-grading*
Lentivirus (HIV1; pLL3.7)	deficient	HEK293T	3	yes
Adenovirus Type 5 (Ad5, Ad5ΔE1GFP)	deficient	a) Hela b) HEK293	a) 3 b) no clearance	a) yes b) no
Adeno-Associated Virus serotype 2 (AAV-2)	deficient (conditionally replicative[¶])	Hela, HEK293	4-5; but inducible by stress and superinfection	no
Vacciniavirus (VVeGFP)	replicative	Vero-B4	Viruses constantly present in sups	no
Modified Vaccinia Ankara (MVA)	reduced (attenuated)	Vero-B4	Viruses constantly present in sups	no

[§]Number of cell passages until no virus-specific nucleic acids were detected in the supernatant.
*The recommendation for downgrading applies to the experimental setup used in this study. The user is advised to test the clearance rate in each expression system individually.
[¶]Can replicate in cells under stress or subjected to coinfection (Berns & Giraud, 1996; Yalkinoglu et al., 1988).

supernatant up to a factor of 3,000 could be observed. Therefore, no proper clearance effect was observed. Thus, downgrading is not possible.

MVA is a variant of VV whose replication capacity has been highly reduced (Meyer et al., 1991). Vero-B4 cells were infected with 2 x 10^4 and 2 x 10^6 pfu/ml of MVA. From PI1 onwards, the supernatant of the cells was sampled and analyzed for the presence of TK gene copies of MVA (Figure 4b). Subsequently, the cells were routinely passaged. MVA gene copies were detectable throughout the monitoring period at constantly increasing levels than used for infection (up to 8.4 x 10^7 gene copies/ml at PI11 vs 3.0 x 10^5 gene copies/ml at PI0) despite continuous washing and passaging. This suggested the production of MVA particles despite its much reduced replication capability. Although MVA is generally known to be highly attenuated, several cell lines other than CEF (chicken embryo fibroblasts) and BHK21 (baby hamster kidney cells) but including Vero-B4 have been shown to be semi-permissive (Okeke et al., 2006). At a titer of > 10^7 MVA gene copies/ml, Vero-B4 cells started to detach from PI18 onwards and eventually died off. This process was slow and lasted for up to 10 days and four passages (Figure 4b, series Vero-B4 2). Therefore, MVA clearance did not occur in viable cells.

Conclusion

The results of this study demonstrate that complete clearance could be achieved in individual experimental setups (Ad5 in Hela and lentivirus (HIV1) in HEK293T; Table 1) in contrast to other virus-host cell systems (Ad5 in Hek293, AAV2 in Hela, HEK293, VV and MVA in Vero-B4) as judged from the presence of vector-specific nucleic acids in the supernatant. Analyzing nucleic acids rather than infectious viruses undoubtedly lead to an overestimation of the amount of viral particles present in the supernatant (Higashikawa & Chang, 2001; Rohr et al., 2002). On the other hand, experiments could be performed with optimized infection rates on which the authors did not set priorities on. The degree and rate of clearance were influenced by several variables in the transduction setups, particularly vector titer and infection protocol. As this study has tested only one viral strain each, the type of strain and insert are likely to have an influence, too. In conclusion, procedures for clearance cannot be predefined for given virus-host systems, and the results presented can serve only as an indication. Each individual experimental setup will have to be tested for clearance before a safe derogation of the containment level will be possible.

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Anthology of Biosafety XIII: Animal Production and Protection—Challenges, Risks, and Best Practices

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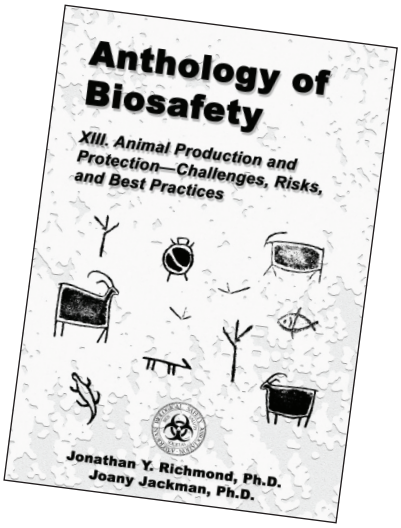

Anthology XIII is certain to capture your interest. Presentations from the February 2011 USDA-ARS 1st International Biosafety & Biocontainment Symposium "Animal Production & Protection: Challenges, Risks, and Best Practices" have been published here, which will forever record their knowledge and perspective on biosafety/biosecurity for the benefit of the biosafety community.

Researchers cannot tackle the problems of Q-fever, bovine encephalitis, monkey pox, or tularemia without working with the infectious agents. Computer models have their role, but the real research occurs in the field and in the laboratories.

Outbreaks of human and animal diseases and particularly outbreaks of food-borne illnesses do not respect political boundaries. Their impact, both in terms of health and economic devastation, is vast and immediate. The recent *E. coli* outbreak in Europe serves to remind us that people expect researchers to be able to quickly identify the cause, the source, and a cure all within a relatively short period of time.

Time issues create risk issues, and it is here where researchers, public health officials, veterinary health officials, and government authorities recognize that safety must remain a concern.

Just as the growth of biosafety knowledge and practices was paramount for conducting non-human primate research, the same need is true for agricultural research. The future availability of a safe and nutritious food supply is critical to social and economic development throughout the world. Diseases that seriously diminish the availability of food sources or that disrupt natural controls have far-reaching consequences. While research dutifully moves to find cures for diseases that haunt us, it is also essential that we not shortchange that which is most basic to our existence.

Contents include, in part: Introduction to Biocontainment and Biosafety Concepts as They Relate to Research with Large Livestock and Wildlife Species; From the Field to the Laboratory in an Animal Disease Outbreak Situation; Working with Biosafety 3 Agents That Interface Across Human, Livestock, and Wildlife Boundaries; Controlling Laboratory Risk…in a Global Biotech Revolution; Liquid Effluent Decontamination Design and Operations; Carcass Disposal for Biocontainment Facilities; and Strategies for Communicating with the General Public About High-containment Laboratories