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Highly attenuated modified vaccinia virus Ankara replicates in baby hamster kidney cells, a potential host for virus propagation, but not in various human transformed and primary cells

Ingo Drexler,[1,2] Karl Heller,[1,2] Britta Wahren,[3] Volker Erfle[1] and Gerd Sutter[1]

[1,2] Institut für Molekulare Virologie, GSF–Forschungszentrum für Umwelt und Gesundheit GmbH[1], and Bavarian Nordic Research Institute GmbH[2], Ingolstädter Landstr. 1, 85764 München-Neuherberg, Germany

[3] Swedish Institute for Infectious Disease Control, Karolinska Institute, 105 21 Stockholm, Sweden

Although desirable for safety reasons, the host range restrictions of modified vaccinia virus Ankara (MVA) make it less applicable for general use. Propagation in primary chicken embryo fibroblasts (CEF) requires particular cell culture experience and has no pre-established record of tissue culture reproducibility. We investigated a variety of established cell lines for productive virus growth and recombinant gene expression. Baby hamster kidney cells (BHK), a well-characterized, easily maintained cell line, supported MVA growth and as proficient expression of the *E. coli lacZ* reporter gene as the highly efficient CEF, whereas other cell lines were non-permissive or allowed only very limited MVA replication. Importantly, no virus production occurred in patient-derived infected primary human cells. These results emphasize the safety and now improved accessibility of MVA for the development of expression vectors and live recombinant vaccines.

The successful worldwide eradication of smallpox via vaccination with live vaccinia virus stimulated research into development of the latter as a highly versatile eukaryotic transient expression vector. Its possible use as a recombinant vaccine for protective immunization against infectious diseases or cancer is particularly attractive (Mackett *et al.*, 1982; Panicali & Paoletti, 1982; for review see Moss, 1996). However, infection with conventional vaccinia virus poses a health risk to both researchers and future patients, particularly immunocompromised individuals. Several attenuated vaccinia virus strains were developed during the smallpox era to reduce possible side effects associated with using live vaccinia virus vaccines (Fenner *et al.*, 1988). In particular, the host range-restricted modified vaccinia virus Ankara (MVA) proved to be extremely attenuated when compared to wild-type vaccinia virus strains (Mayr *et al.*, 1975, 1978; Werner *et al.*, 1980). In clinical trials, MVA was administered without significant side-effects to about 150000 individuals, including many considered at risk for the conventional smallpox vaccination (Stickl *et al.*, 1974; Mahnel & Mayr, 1994). MVA was originally derived from the vaccinia strain Ankara by over 570 serial passages in primary chicken embryo fibroblasts (CEF) severely compromising its capacity to replicate in mammalian cells (Mayr *et al.*, 1975). Further studies revealed that six major deletions had occurred in the DNA of the attenuated virus compared to the parental genome (Meyer *et al.*, 1991). Virus replication is blocked late in morphogenesis in non-permissive cells, but importantly, viral and recombinant protein synthesis is unimpaired at early and late times after infection (Sutter & Moss, 1992). Hence, replication-deficient recombinant MVA was established as an exceptionally safe viral vector (Sutter & Moss, 1995; Moss *et al.*, 1996) and can be used in Germany and the USA under biosafety level 1 conditions (Stellungnahme der Zentralen Kommission für Biologische Sicherheit, AZ 6790-10-14, Berlin, FRG, 5/1997; Moss, 1996). MVA recombinants expressing bacteriophage T7 RNA polymerase genes have been constructed (Wyatt *et al.*, 1995; Sutter *et al.*, 1995) and used successfully as expression systems for reverse genetics of RNA viruses (Collins *et al.*, 1995; Schneider *et al.*, 1997; Baron & Barrett, 1997). When tested in animal model systems recombinant MVA have been shown to be avirulent, yet protective vaccines for immunization against viral diseases and cancer (Sutter *et al.*, 1994; Hirsch *et al.*, 1996; Wyatt *et al.*, 1996; Carroll *et al.*, 1997). Despite, or rather because of the advantages conferred by the severe host range restriction of MVA, its propagation is not trivial, being restricted to primary CEF cells. The establishment and maintenance of CEF cultures requires experience in preparing primary tissue culture and depends on egg material from chicken kept under special pathogen-free conditions. In addition, CEF cultures survive only a few passages and weekly *de novo* preparations are

Author for correspondence: Gerd Sutter.
Fax +49 89 3187 3329. e-mail sutter@gsf.de

0001-5149

Table 1. MVA one-step growth in established cell lines

Cells infected with 10 IU per cell MVA-LZ were harvested after the adsorption period (0 h) or 24 h after infection. Virus titres (T_0, T_{24}) were determined by backtitration on CEF. Virus multiplication is demonstrated by the ratios of the average virus titres determined after 24 h over the titres at the beginning of infection (T_{24}/T_0) in two independent titrations from a single one-step growth experiment. In two additional one-step growth experiments T_{24}/T_0 values of 136 and 250 for CEF, 42 and 56 for BHK, and 4 and 7 for CV-1 were calculated.

Cell origin	Name	T_{24}/T_0
Animal		
Chicken embryo	CEF	316
Baby hamster kidney	BHK	66
Monkey kidney	CV-1	32
Monkey transformed B-cell	MIB	< 1
Human		
Cervix carcinoma	HeLa	5
Melanoma	SK 29 MEL 1	< 1
Transformed kidney	293	4
Embryonic lung	LC 5	< 1
Astrocytoma	85 HG 66	< 1
Glioblastoma	U 138	< 1
T-cell lymphoma	C 8166	< 1
T-cell lymphoma	HUT 78	< 1
Transformed B-cell	SY 9287	< 1

required. Therefore, a cell line that is easily and stably maintained would not only be very useful for experimental propagation of MVA vectors, but also represent a desirable tool for making recombinant MVA vaccines, where ease, low costs, reproducibility and a pre-established safety record of production are essential.

In this study, immortalized and primary human and animal cell lines (Table 1) were investigated for productive growth of a recombinant MVA which encodes the *E. coli lacZ* reporter gene under the control of the vaccinia virus late promoter P11 (MVA-LZ; Sutter & Moss, 1992). In addition, a vaccinia strain Copenhagen recombinant virus encoding the *E. coli lacZ* gene under the control of the early/late promoter P7.5 (VV-LZ; courtesy of Robert Drillien, University of Strasbourg, France) was used. MVA-LZ or VV-LZ were routinely propagated and titred by endpoint dilution in CEF or MA104 cells, respectively, to obtain the $TCID_{50}$. The animal cell cultures investigated were: primary chicken embryo fibroblasts (CEF); baby hamster kidney (BHK; gift from Lothar Schneider, Bundesforschungsanstalt für Viruskrankheiten der Tiere, Tübingen, Germany); the monkey kidney cell lines MA104 (Rhône Mérieux, Lyon, France) and CV-1 (ATCC CCL 70); and a herpes papiovirus-transformed monkey lymphoblastoid B-cell line, MIB. The human cell lines were: T-cell lymphoma C8166 (Medical Research Council, AIDS Reagent Project, Repository Reference ADP013) and HUT78 (Medical Research Council, AIDS Reagent Project, Repository Reference ADP002); glioblastoma U138MG (Medical Research Council, AIDS Reagent Project, Repository Reference ADP028) and astrocytoma 85HG-66 (Brack-Werner *et al.*, 1992); embryonal lung fibroblasts LC5 (Medical Research Council, AIDS Reagent Project, Repository Reference ADP026; Mellert *et al.*, 1990); cervix carcinoma HeLa (ATCC CCL 2); Epstein–Barr virus-transformed lymphoblastoid B-cell line SY9287 (a gift from Robert Drillien, University of Strasbourg, France); and melanoma SK29MEL1 (a gift from Thomas Wölfel, University of Mainz, Germany). Primary human cells were: fibroblasts (HF) obtained from fresh skin biopsy material; peripheral blood mononuclear cells (PBMC) isolated by Ficoll gradient purification; monocytes (MO) or dendritic cells (DC) separated by anti-CD14 cell rosetting followed by Percoll gradient purification or magnetic sorting (MACS), respectively. All cell cultures were grown in RPMI 1640 supplemented with 10% foetal calf serum (FCS) in a humidified air–5% CO_2 atmosphere. The DC culture medium additionally contained IL4 (400 U/ml) and GM-CSF (50 ng/ml).

To determine low-multiplicity growth profiles, virus multiplication was monitored after infecting cell monolayers and suspension cultures with 0·05 infectious units (IU) MVA-LZ per cell, as described previously (Meyer *et al.*, 1991). Additionally, one-step growth of MVA-LZ was analysed infecting cells at an m.o.i. of 10 IU. For all infection experiments either 10^6 cells in suspension or confluent monolayers from one well of six-well tissue culture plates were used per time point. After virus adsorption for 45 min at 37 °C for low-multiplicity growth or 30 min at 4 °C for the one-step growth experiments, the inoculum was removed. The infected cells were washed three times with RPMI 1640 and incubated with fresh RPMI 1640 medium containing 10% FCS at 37 °C in a 5% CO_2 atmosphere. At multiple time-points post-infection (p.i.) infected cells were harvested and virus was released by freeze–thawing and brief sonication. Serial dilutions of the resulting lysates were plated on confluent CEF monolayers grown in 96-well plates as replicates of eight. For histochemical staining of β-galactosidase-producing cells, medium was removed 48 h p.i., and then cells were washed twice with PBS and briefly fixed in 0·2% glutaraldehyde–2% formaldehyde. After washing, cells were incubated in a staining solution containing 0·6 mg/ml chromogenic substrate 5-bromo-4-chloro-3-indolylβ-galactopyranoside (X-Gal, Boehringer Mannheim), 5 mM ferrocyanide and 5 mM ferricyanide and 2 mM $MgCl_2$ in PBS at 37 °C for 2 h. Microscopic analysis monitoring for wells containing blue stained cells allowed the determination of virus titres as $TCID_{50}$/ml.

Previous work demonstrated that a variety of mammalian cells lines (of human, bovine, equine, canine or rodent origin) are non-permissive for MVA replication. Only the African green monkey kidney cell line MA104 supported MVA growth under low-multiplicity growth conditions (Meyer *et al.*, 1991). In one-step growth experiments performed for this

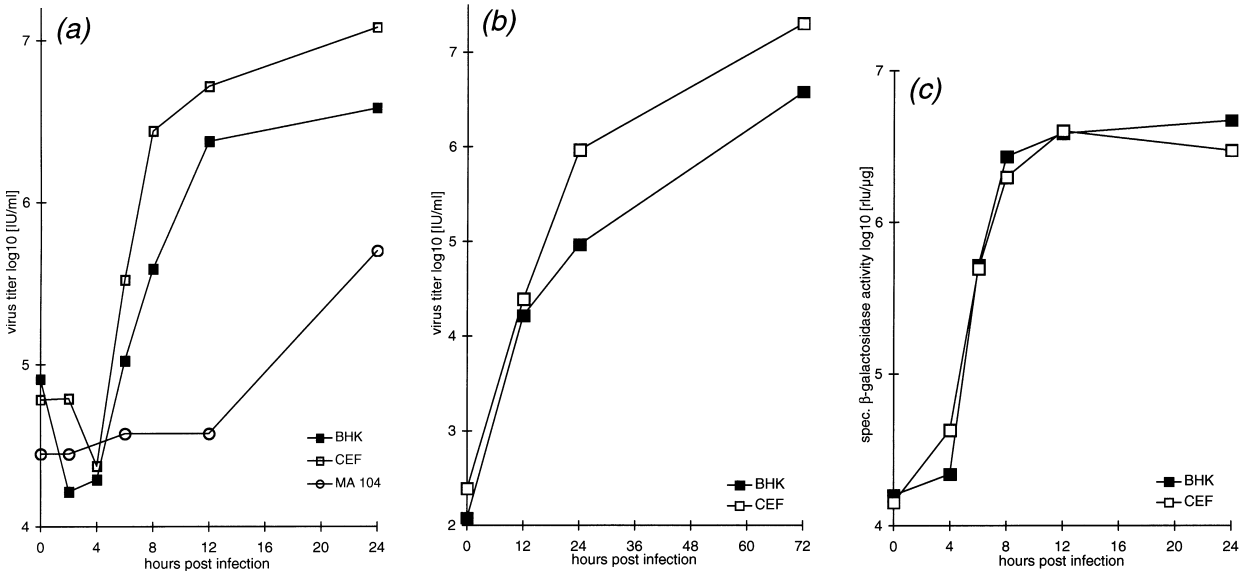


Fig. 1. MVA growth and recombinant gene expression in BHK cells. One-step growth curves were established after infection of BHK, MA104 or CEF cells with 10 IU per cell MVA-LZ (*a*). Low-multiplicity growth of MVA was analysed after infection of BHK cells or CEF with 0·05 IU per cell MVA-LZ (*b*). Cell cultures were harvested at the indicated time-points after infection. Virus titres were determined by titration on CEF. Synthesis of recombinant *β*-galactosidase in BHK cells and CEF was compared (*c*). Cells were infected with 10 IU per cell MVA-LZ and harvested at multiple time-points after infection. Cytoplasmic extracts were prepared and specific *β*-galactosidase activities (relative light units/µg total protein) were determined.

study, MVA could also be propagated in the kidney cell lines CV-1 and BHK from monkey and hamster, respectively. While CV-1 and MA104 cells produced at best about one-tenth the amount of virus compared to CEF cells, BHK were far more permissive (Table 1). That BHK cells produced significant virus titres was worth further investigation. Very similar virus replication profiles were observed with multiple time-point one-step growth kinetics in BHK and CEF cells (Fig. 1*a*). Even the timing of the post-adsorption lag phase is identical in both cell lines, whereas productive infection of MVA in MA104 cells leads to rather delayed and reduced increase of virus titres. Experiments using low multiplicities of infection also revealed similar kinetics of MVA replication in BHK and CEF cells, particularly within the first 12 h (Fig. 1*b*). These data suggest that assembly of mature virions is not significantly different or delayed in BHK cells compared to CEF cells. Interestingly, low-dose MVA infection of BHK cell monolayers resulted in formation of typical foci of infected cells which remained attached to the tissue culture plates (data not shown). Expression of the recombinant gene *lacZ* under the control of the vaccinia virus late promoter P11 was also monitored, including detailed kinetics of *β*-galactosidase production in BHK and CEF cells infected with 10 IU per cell of MVA-LZ. After incubation of infected cultures for various times (0, 4, 6, 8, 12 and 24 h), the specific enzyme activity in the prepared cytoplasmic extracts was determined (Miller, 1972) and proved to be almost identical in both BHK and CEF (Fig. 1*c*). These data support the use of BHK cell monolayers for isolation of recombinant MVA expressing the *lacZ* marker gene.

Of particular interest for *in vivo* applications of live MVA vaccines in humans is its replication profile and recombinant gene expression in potential target cells. Although shown to be replication-deficient in multiple transformed cell lines of human origin (Table 1) investigation of MVA virus production in primary human cells would provide another stringent test for its clinical safety. Primary cells with particular capacities in antigen-presentation such as DC or blood monocytes could represent important target cells for clinical trials using *ex vivo* infection of patient cells for immune therapy. After *in vitro* infection, these cells would produce and present the desired antigens and either be retransferred to the patient to stimulate antigen-specific immune responses or used for *in vitro* induction or amplification of T-cells which can than be adoptively transferred (Boon *et al.*, 1995; Rosenberg, 1996; Girolomoni & Ricciardi-Castagnoli, 1997). The approval of such clinical protocols might be significantly accelerated if any productive replication of the vector virus in target cells can be excluded. Blood-derived cells or primary skin fibroblasts representing ideal target cells for such clinical trials were used in our experiments. Although abortive infection of primary human macrophages or DC has been demonstrated even with replication-competent vaccinia virus strains (Broder *et al.*, 1994; Bronte *et al.*, 1997), PBMC and HF are reasonably permissive for vaccinia virus multiplication. In contrast, by analysing virus multiplication under one-step growth conditions we could demonstrate the inability of MVA to replicate in all primary human cells tested (Fig. 2*a*). In addition to the detailed kinetics of virus growth, histochemical *β*-galacto-

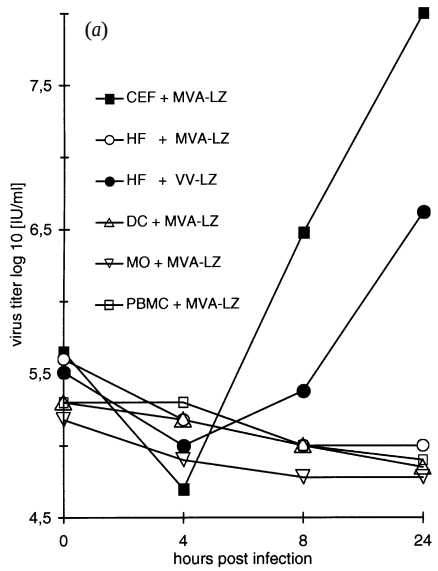
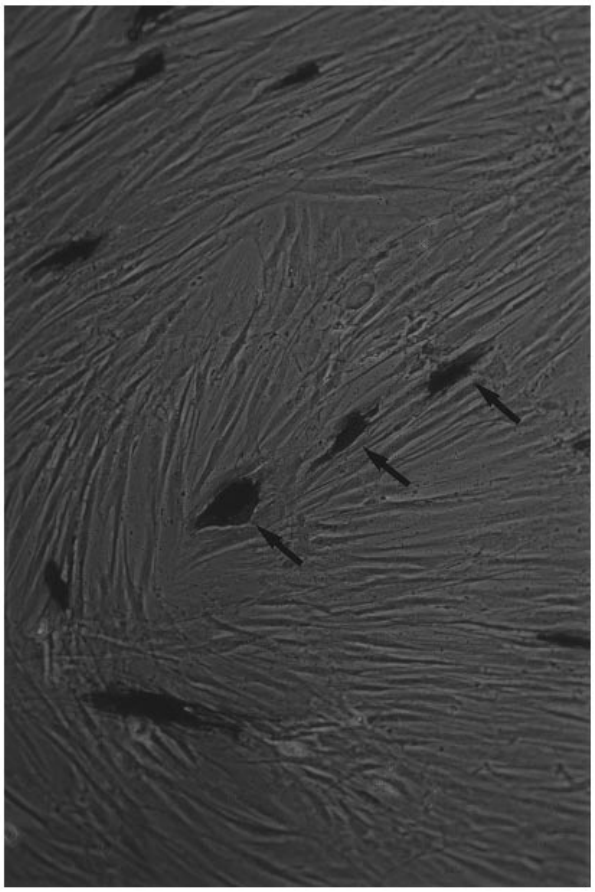
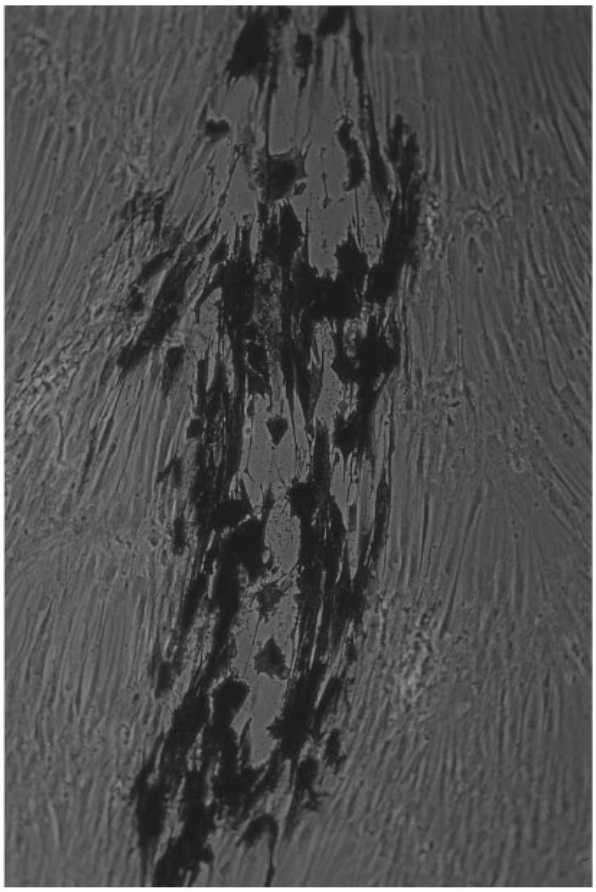


Fig. 2. MVA multiplication in primary human cells. One-step virus growth curves are shown for CEF cells and for primary human cells (HF, DC, MO and PBMC) (*a*). Virus growth was analysed after infection of cells with 10 IU per cell MVA-LZ or VV-LZ. Virus titres in cells harvested at 0, 4, 8 and 24 h after infection were determined by back-titration on CEF. (*b*, *c*) Micrographs of HF monolayers infected with a low m.o.i. of MVA-LZ (*b*) and VV-LZ (*c*). At 48 h p.i., the cells were fixed and stained histochemically with X-Gal. Three single cells infected with MVA-LZ are marked by arrows. One single virus plaque formed by VV-LZ is shown.

(*b*)



(*c*)



sidase-specific staining of HF monolayers infected at low-multiplicity confirmed that MVA infection was non-permissive for productive replication but allowed efficient expression of recombinant genes regulated by the vaccinia virus late promoter P11 (Fig. 2*b*). In contrast, VV-LZ replicated well in HF forming large lytic plaques in the cell monolayer (Fig. 2*c*).

In summary, the screening for virus production in various human and animal cells recommends BHK cells as an accessible alternative to primary CEF cells for routine MVA virus propagation and manipulation. BHK cell lines are routinely used in many laboratories, and even licensed in some cases for vaccine production. Although high virus titres were obtained from the BHK cell line, there was no evidence of significant MVA virus production in various other human or animal cell lines chosen for this investigation. Another important finding was the complete inability of MVA to productively replicate in all primary human cells tested. Together with the existing evidence that MVA is a very safe live vaccine in humans, these results make the handling and testing of recombinant MVA virus much more accessible to a variety of experimental and clinical research projects aimed at developing prophylactic vaccination and therapeutic treatment of infectious diseases and cancer.

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