Properties of human foamy virus relevant to its development as a vector for gene therapy

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The Spumaviridae (foamy viruses) are increasingly being considered as potential vectors for gene therapy, yet little has been documented of their basic cell biology. This study demonstrates that human foamy virus (HFV) has a broad tropism and that the receptor for HFV is expressed not only on many mammalian, but on avian and reptilian cells. Receptor interference assays using an envelope-expressing cell line and a vesicular stomatitis virus/HFV pseudotype virus demonstrate that the cellular receptor is common to all primate members of the genus. The majority of foamy virus particles assemble and remain sequestered intracellularly. A rapid and quantitative method of assaying foamy virus infectivity by reverse transcriptase activity facilitates the use of classical protocols to increase infectious virus titres in vitro to $\geq 10^6$ TCID/ml.

Introduction

Foamy viruses are so called because of the characteristic ‘foamy’ appearance of the cytopathic effect (CPE) induced in cells following in vitro culture of the virus. They are apathogenic in accidentally infected humans (Schweizer et al., 1997) and naturally or experimentally infected animals (Schmidt et al., 1997), and early reports of natural infection in various parts of the world (Achong & Epstein, 1978) have not been confirmed using a range of modern techniques on much larger sample sizes (Ali et al., 1996). It is, therefore, not surprising that foamy viruses have remained, until recently, the least studied of the Retroviridae. However, they are emerging from obscurity to be considered as potential retrovirus vectors.

The human foamy virus (HFV) belongs to a group of complex retroviruses which encode three structural genes (gag, pol and env) in addition to three accessory open reading frames (bel-1, -2, -3) located in the 3’ end of the genome. Of these only bel-1 (now called his), which encodes the transcriptional transactivator (Tas), is essential for virus replication (Baunach et al., 1993). The Gag, Pol and Env proteins are synthesized independently (Enssle et al., 1996; Lochelt & Flügel, 1996; Yu et al., 1996b) and, therefore, in terms of vector consideration, can be provided in trans on three different plasmids to create stable packaging cell lines, reducing the possibility of generating replication-competent helper virus (Bieniasz et al., 1997). First studies in which foamy viruses have been developed as gene delivery vehicles (Schmidt & Rethwilm, 1995; Russell & Miller, 1996; Bieniasz et al., 1997) have involved replacing some or all of the bel genes of pHSRV, an infectious molecular clone of HFV (Rethwilm et al., 1990), with a reporter gene to assess transduction. Replication-competent vectors induced the characteristic CPE when transfected into BHK cells (Schmidt & Rethwilm, 1995), while replication- incompetent vectors transduced a variety of cell lines (Russell & Miller, 1996) at maximum titres of $10^8$ transducing units/ml (Schmidt & Rethwilm, 1995; Bieniasz et al., 1997).

This paper reports some basic aspects of foamy virus infection relevant to future vector development. The aims of this study were to find a simple assay of infectivity with which to optimize infection conditions and increase virus titre, to consider foamy virus tropism and, by means of an HFV envelope-expressing cell line, exploit the phenomenon of receptor interference to determine whether different foamy viruses share a common cellular receptor. This is based on the observation that chronically infected cell lines are resistant to superinfection by homologous viruses utilizing the same receptor. The identification of cellular receptors which mediate virus attachment and entry is a fundamental step in the investigation of the molecular events involved during retrovirus infection. Although several retrovirus receptors have...
been identified (Weiss & Tailor, 1995), nothing is known of the cell surface molecules which enable foamy viruses to enter cells. The BHK Env-expressing cell line and the vesicular stomatitis virus (VSV) pseudotypes expressing foamy virus envelopes described in this paper constitute useful tools with which to investigate further the mechanism of HFV attachment to cells.

Methods

■ Cell lines. Most cell lines used in the study (Table 1) were cultured in DMEM plus 10% foetal calf serum (FCS), 25,000 units/ml penicillin and 25 mg/ml streptomycin. The exceptions were the cell lines MPK and QT6, which were grown in medium containing 20% FCS and 10% FCS +2% chicken serum, respectively.

■ BHK-Env cells. A eukaryotic expression plasmid, pEnv3.1, in which HFV envelope expression is driven by a simian virus 40 early promoter was constructed using the expression plasmid pSG5 (Stratagene). BHK-Env cells.

<table>
<thead>
<tr>
<th>Table 1. Sensitivity of cell lines to HFV infection</th>
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<tr>
<td>Cells were infected with HFV and at 48 h post-infection monolayers were immunoperoxidase-stained using an HFV-positive serum, as described. Cells were scored visually according to the proportion of cells which expressed viral antigen. + + + +, &gt; 50% positive; + + +, 10–50% positive; + +, 1–10% positive; +, &lt; 1% positive; −, negative.</td>
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<tr>
<th>Cell line</th>
<th>Species/tissue of origin</th>
<th>Sensitivity to HFV infection</th>
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<tbody>
<tr>
<td>HEL</td>
<td>Human lung fibroblasts</td>
<td>+ + + +</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human epithelium</td>
<td>+</td>
</tr>
<tr>
<td>RD</td>
<td>Human rhabdomyosarcoma</td>
<td>+ +</td>
</tr>
<tr>
<td>HT29</td>
<td>Human colorectal epithelium</td>
<td>+</td>
</tr>
<tr>
<td>HT1080</td>
<td>Human fibrosarcoma</td>
<td>+ + + +</td>
</tr>
<tr>
<td>293</td>
<td>Human epithelium</td>
<td>+ + + +</td>
</tr>
<tr>
<td>COS-7</td>
<td>African green monkey kidney</td>
<td>+</td>
</tr>
<tr>
<td>WOP</td>
<td>Mouse fibroblasts</td>
<td>+ + + +</td>
</tr>
<tr>
<td>LMTk</td>
<td>Mouse fibroblasts</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>Mouse fibroblasts</td>
<td>+ +</td>
</tr>
<tr>
<td>BHK-21</td>
<td>Hamster kidney fibroblasts</td>
<td>+</td>
</tr>
<tr>
<td>CHO K1</td>
<td>Hamster ovary epithelium</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>Rat-1</td>
<td>Rat fibroblasts</td>
<td>+ + + + + + +</td>
</tr>
<tr>
<td>Mv.1.Lu</td>
<td>Mink lung fibroblasts</td>
<td>+ +</td>
</tr>
<tr>
<td>Sp1K</td>
<td>Dolphin kidney epithelium</td>
<td>+ + + +</td>
</tr>
<tr>
<td>PAE</td>
<td>Pig aortic fibroblasts</td>
<td>+ + + +</td>
</tr>
<tr>
<td>PK15</td>
<td>Pig kidney epithelial</td>
<td>+ + + +</td>
</tr>
<tr>
<td>St Iowa</td>
<td>Swine testes</td>
<td>+ + + + + + + +</td>
</tr>
<tr>
<td>MPK</td>
<td>Minipig kidney</td>
<td>+ + + + + + + + +</td>
</tr>
<tr>
<td>Munjack</td>
<td>Indian munjack fibroblasts</td>
<td>+</td>
</tr>
<tr>
<td>Avian</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QT6</td>
<td>Quail fibroblasts</td>
<td>+ + + + + + + + +</td>
</tr>
<tr>
<td>CBGQ</td>
<td>Goose fibroblasts</td>
<td>+ + + + + + + + + + +</td>
</tr>
<tr>
<td>Reptilian</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgH2</td>
<td>Iguana epithelium</td>
<td>+ + + + + + + + + + + + + +</td>
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21 cells were co-transfected with 20 µg pEnv3.1 and 1 µg pBabepruro (Morgenstern & Land, 1990) using calcium phosphate co-precipitation. Single cell clones were selected in medium containing 2.5 µg/ml puromycin. Foamy virus-immune chimpanzee serum was used to identify envelope-expressing cells (Moebes et al., 1997). Functional expression of the HFV envelope was confirmed by co-cultivation of BHK-Env or BHK-21 cells with primary human embryonic lung fibroblasts for 24 h followed by staining with 0.5% methylene blue and 0.1-% basic fuchsin in methanol to detect cell fusion (data not shown).

■ Wild-type viruses. Virus isolates used were as follows: HFV (Achong et al., 1971), simian foamy viruses SFV-1 and SFV-2 different serotypes from rhesus macaques (Stiles, 1968), SFV-3 from an African green monkey (Stiles, 1968; Swack et al., 1970) and the chimpanzee isolates, SFV-6 and SFV-7 (Hoores et al., 1973).

■ Production of VSV (HFV) pseudotype virus. VSV (HFV) pseudotypes were generated as previously described (Schnitzer, 1982). BHK-21 cells or BHK-Env cells were acutely infected with VSV (Indiana serotype) at approximately 1 p.f.u. per cell. After 1 h the inoculum was removed and replaced with 4 ml fresh medium. The following day, culture supernatant was harvested, passed through a 0.45 µm filter and stored in 0.5 ml aliquots at −70 °C. For VSV pseudotype titration, 50 µl pseudotype stock was incubated with 50 µl of 1:4 diluted sheep anti-VSV serum for 1 h at 37 °C. Non-neutralized controls were incubated with DMEM/10% FCS. BHK-21 or BHK-Env cells, seeded at 8 x 10⁴ cells per well in 24-well plates, were inoculated with 10-fold serial dilutions of virus or virus serum mixture. After 1 h incubation, the inoculum was removed and the cells overlaid with 250 µl agarose medium. After 5 min at room temperature, to permit solidification of the agarose, cells were cultured for 48 h. Two hundred and fifty µl of 0.1% neutral red in PBS was added to each well, and after 1 h plaques were observed microscopically.

■ Focal immunoassay (FIA). HFV infection in diverse cell lines was assayed by FIA (Bieniasz et al., 1995). Briefly, virus was added in 10-fold serial dilutions to cells seeded at 5 x 10⁴ cells per well in 24-well plates. Forty-eight hours post-infection, cells were washed with PBS and fixed for 10 min with 500 µl of a 1:1 acetone–methanol solution at −20 °C. On removal of the fixative, cells were air-dried for 5 min, washed in PBS/1% FCS and incubated for 45 min at room temperature with 200 µl anti-HFV antibody (HFV-positive serum from an accidentally infected laboratory worker; Schweitzer et al., 1997) diluted 1:200 in PBS/1% FCS. Unbound antibody was removed by washing three times in PBS/1% FCS and the cells were incubated for 45 min at room temperature with 200 µl of 1:100 anti-human IgG Fab(r) fragments conjugated to horseradish peroxidase in PBS/1% FCS. Cells were washed twice in PBS before addition of 300 µl of 0.3 mg/ml 3’-diaminobenzadine, 0.1% H₂O₂ in PBS for 15 min, and examined microscopically for stained foci of infection.

■ Reverse transcriptase (RT) assay. RT activity was assayed by the C-type-RT [manganese (Mn²⁺)-dependent] and Lenti-RT [magnesium (Mg²⁺)-dependent] activity assays from Cavidi Tech (Ekstrand et al., 1996; Malmsten et al., 1998). Briefly, polyadenylic acid (poly(rA)) was covalently coupled to the wells of a 96-well microtitre plate and used as a template for the incorporation of the nucleotide analogue 5-bromo-2’-deoxyuridine 5’triphosphate (BrdUTP), with oligo(dt)₇ as a primer. Incorporated BrdUMP product was quantitatively detected immunologically using alkaline phosphatase-conjugated anti-BrdU monoclonal antibody and para-nitrophenyl phosphate as substrate for colorimetric detection at 405 nm after 1 h incubation.

■ Enhancement of HFV titre. BHK-21 cells were seeded at 1 x 10⁵ cells/ml in 25 cm² flasks (6 ml per flask), infected with HFV at an m.o.i.
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![Fig. 1. Optimization of HFV infection in BHK-21 cells.](image)

(A) BHK-21 cells were infected at an m.o.i. of 0.005 and supernatant was collected each day post-infection and analysed for RT activity by the C-type RT assay. Cells were passaged on days 3 and 6. (B), (C) Infection of BHK-21 cells with HFV at a range of m.o.i. (0–0.1). At 7 days post-infection supernatant was collected and (B) RT activity determined by the C-type RT assay. (C) Number of infectious units produced per cell calculated by FIA and total cell count.

of between 0 and 0.1 in 1 ml medium and incubated at 37 °C in a 5% CO₂ environment. When using Lipofectin (Gibco BRL) to enhance adsorption to cells, virions were complexed with 20 µl lipid for 1 h at room temperature prior to maintenance under normal conditions. Cells were observed for CPE each day and passaged 10 at confluence. Virus was harvested from cells and cell supernatant using one or a combination of the following methods. (i) Infected cells were harvested in a small volume (2 ml per 25 cm² flask) of fresh medium and subjected to three cycles of freezing on dry ice and thawing at 37 °C. (ii) Cellular debris from either supernatant or freeze–thawed infected cells was removed by centrifugation at 500 g for 5 min or by filtration (0.45 µm, Millipore). (iii) Virus supernatant was harvested and concentrated 200-fold by centrifugal ultrafiltration (Centricon, Amicon).

**HFV infection of cell lines.** Cells were seeded in either 6-well plates or in 25 cm² flasks. When 30–50% confluent (approx. 1–5 × 10⁵ cells per well or 3 × 10⁵–2 × 10⁶ cells per flask) cultures were inoculated with 3 × 10⁵ focus-forming units (f.f.u.) virus per 25 cm² flask or 1 × 10⁵ f.f.u. per well in 6-well plates. Forty-eight hours post-infection cells were assayed for virus antigen by FIA.

**Receptor interference.** BHK-21 cells or BHK-Env cells were seeded in 24-well plates and the following day infected with 10-fold serial dilutions of SFV-1, SFV-2, SFV-3, SFV-4, SFV-7 and HFV. After 48 h, infectious titres were determined by FIA.

**Results**

**RT assay and enhancement of virus titre**

A C-type-RT assay (Cavidi Tech) which contains the divalent cation Mn²⁺ was compared with an identical assay for human immunodeficiency virus (HIV), the Lenti-RT, which uses Mg²⁺ cations. The Mn²⁺-based assay detected higher enzyme activity, down to 10 infectious virions/ml, as determined by FIA (data not shown). This assay provided a quick and reliable means of determining the time post-infection that virions were optimally released from infected cells. Cell-free supernatants (300 µl) were collected each day post-infection from BHK cells infected at an m.o.i. of 0.005 and RT activity assayed by the C-type-RT assay. The highest RT activity was produced on day 7 (Fig. 1A), while syncytium formation was observed from day 4 onwards and by day 9 there was little cell growth and extensive CPE in the cultures.

Cationic liposomes were used to maximize the virus–cell interaction. Thus, BHK-21 cells were infected at an m.o.i. of 0.005 in the presence and absence of Lipofectin. At 4–7 days post-infection there was no difference in CPE between the two cultures and virus titres were similar.

To determine the effect of m.o.i. on virus yields, BHK-21 cells were infected at an m.o.i. of 0–0.5, 0–1 virions per cell. Supernatants were harvested on day 7 post-infection at optimal RT activity. Total cell number, RT activity in cell-free supernatant and extracellular virus titre (by FIA) were determined (Fig. 1B, C). Between an m.o.i. of 0 and 0.05, the higher the m.o.i., the greater the RT activity and virus titre. There was a sixfold increase in RT activity at an m.o.i. of 0.05 compared with infection at an m.o.i. of 0.001 (Fig. 1B). Cells infected at an m.o.i. of 0.1 produced a marked CPE on day 4, and by day 7 there were few viable cells remaining to support virus replication and although RT activity was detected, no infectious virus was observed by FIA (Fig. 1C). Fig. 1 shows that the optimal m.o.i. for infection was between 0.01 and 0.05.
Fig. 2. Receptor interference assays. HFV-infected BHK-21 cells and BHK-Env cells were detected by immunoperoxidase staining using HFV-positive human antiserum (A–H) or pooled foamy virus-positive macaque sera (I–P): SFV-6-infected BHK-21 cells (A), SFV-6-infected BHK-Env cells (B), SFV-7-infected BHK-21 cells (C), SFV-7-infected BHK-Env cells (D), HFV-infected BHK-21 cells (E), HFV-infected BHK-Env cells (F), uninfected BHK-21 cells (G), uninfected BHK-Env cells (H), SFV-1-infected BHK-21 cells (I), SFV-1-infected BHK-Env cells (J), SFV-2-infected BHK-21 cells (K), SFV-2-infected BHK-Env cells (L), SFV-3-infected BHK-21 cells (M), SFV-3-infected BHK-Env cells (N), uninfected BHK-21 cells (O) and uninfected BHK-Env cells (P).
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To facilitate virus release, cultures showing extensive CPE were subjected to lysis by freeze–thawing. Following removal of the cellular debris the resulting virus titre was 10^4–10^5 compared to 10^2–10^3 for virus-containing supernatant harvested from infected cells. Using a Centricon filter (Amicon/ Millipore) with a pore size of 100 kDa it was possible to concentrate 200 ml (titre 5 x 10^5) HFV virus-containing supernatant to 1 ml with a titre of 1 x 10^6, with no loss of infectivity to the filtrate. A combination of all the above-mentioned procedures provided HFV stocks with a titre of > 10^6.

HFV infection of cell lines

The results of infecting diverse cell lines with HFV are shown in Table 1. Inoculation of a variety of mammalian, two avian and one reptilian cell lines resulted in immunoperoxidase staining of virus antigen by FIA, while mock-inoculated control cells remained unstained. These data demonstrate that the HFV receptor is not only widely distributed among mammalian cell lines, but it is also present on cells of avian and reptilian origin.

Receptor interference assays

To investigate whether SFVs use the same cell surface receptor as HFV, the infectivity of isolates either closely related (SFV-6, SFV-7) or more distantly related (SFV-1, SFV-2, SFV-3) to HFV were determined using FIA, with BHK-21 and BHK-Env cells as target cells (Figs 2 and 3). None of the sera used stained BHK-Env cells in the absence of virus infection. Immunoperoxidase staining of BHK-21 and BHK-Env cells (Fig. 2), each inoculated with SFV-6 (Fig. 2 A, B), SFV-7 (Fig. 2 C, D), HFV (Fig. 2 E, F), SFV-1 (Fig. 2 I, J), SFV-2 (Fig. 2 K, L) or SFV-3 (Fig. 2 M, N) showed fewer infected BHK-Env cells compared with BHK-21 cells. In fact, end-point titres, determined using BHK-Env cells (used within 1 month of cloning), were 500–1500-fold lower than on BHK-21 cells (Fig. 3), suggesting that these viruses use the same cell surface receptor as HFV. To confirm that this phenotype was determined at the level of entry, a VSV pseudotype bearing the HFV envelope was generated by passage of VSV in BHK-Env cells. After neutralization with anti-VSV serum, a pseudotype titre of 2 x 10^3 p.f.u./ml was observed on BHK-21 cells, whereas no plaques were observed when BHK-Env cells were inoculated with 20 µl VSV (HFV). Thus, VSV (HFV) was at least 40-fold less infectious on BHK-Env cells compared with BHK-21 cells (Fig. 3). In contrast, when non-pseudotyped VSV was titrated on BHK-21 and BHK-Env target cells, an equal number of plaques was observed, and no infectivity was observed after neutralization with anti-VSV serum. These results suggest that HFV and distinct SFV neutralization serotypes share a common saturable receptor on BHK-21 cells.

Discussion

Foamy viruses bud from intracellular membranes and remain sequestered in the cell, resulting in low extracellular titres and constituting a practical disadvantage to their investigation and exploitation as vectors. Traditional methods for virus titre enhancement were tested and the effects monitored using a commercially available RT assay designed for C-type retroviruses, the RTs of which are Mn^{2+}-dependent. Cation dependence of the foamy virus RT is not exclusive, but the Mg^{2+} assay for HIV-1 proved less sensitive, in agreement with previous published data (Kögel et al., 1995). The greatest RT activity, an indication of virus particles, in cell-free supernatant of infected cells occurred 7 days post-infection. Thus, maximal release of virions from infected cells occurs approximately 72 h after the first syncytia were observed. The optimal m.o.i. for infection was found to be between 0·01 and 0·05. A lower m.o.i. did not provide a productive infection, and a higher m.o.i., although producing CPE on day 4, caused extensive cell death by day 7 and although the RT activity remained high the end-point titre, determined by serial dilution of the supernatant, was greatly reduced. This suggests that RT production is not a limiting factor in the production of replication-competent virions from infected cells or alternatively that RT is more stable than the virus.

In comparison with previous reports of HIV and ecotropic murine leukaemia virus infections which were enhanced by surface charge reduction (Faller & Baltimore, 1984; Innes et al., 1990; Konopka et al., 1990), the cationic liposome formulation of Lipofectin was utilized to attempt to enhance the HFV infection rate by reducing surface charge. Lipofectin had no effect on HFV production, demonstrating that virion surface charge is not a limiting factor during HFV infection.
During foamy virus infection in vitro, the majority of virus particles remain within host cells, where they bud from intracellular membranes (Bodem et al., 1997). Release of virions from infected cells by disruption of the cell membrane by freeze–thawing, removal of cell debris by filtration or low speed centrifugation and virion concentration by ultrafiltration increased HFV titre from 10^3 to 10^4 virions/ml. It has been reported that HFV titres of approx. 10^6 can also be obtained by transfection of BHK-21 cells with human spumaretrovirus and placing the CPE-producing lysates onto low passage HEL-299 cells (Yu & Linial, 1993). Further increases may require a better understanding of the role of the HFV endoplasmic reticulum retrieval signal present in the envelope glycoprotein (Goepfert et al., 1997).

In this paper, analysis of a variety of cell lines from diverse species confirmed that the receptor for HFV is widely expressed in mammals and is also present in birds and reptiles. In addition to the cell lines studied here, HFV has also been reported to productively infect cell lines derived from rabbits, cows, dogs, cats, sheep and chickens (Hooks & Gibbs, 1975; Russell & Miller, 1996) as well as many cell types of human origin including cell lines of epithelial, neuronal, myeloid and fibroblastoid origin (Hooks & Gibbs, 1975; Hooks & Dentrick-Hooks, 1981; Neumann-Haefelin et al., 1983; Yu et al., 1996a). The ubiquitous nature of the HFV receptor suggests that it may perform an essential conserved cellular function in eukaryotes. The apparent absence of a cell line refractory to foamy virus infection has hampered attempts to clone the cellular receptor by genetic means. However, the ubiquitous presence of the receptor in cell lines of diverse lineage and derived from both mammalian and invertebrate species might indicate that the receptor is not protein in nature. Nevertheless using a BHK-21-derived cell line constructed to express HFV envelope, we demonstrate from receptor interference assays that the receptor is saturable and is shared by several different primate foamy virus isolates. Overall, these results indicate that (i) foamy viruses can be produced in reasonable quantities, (ii) foamy viruses are concentratable without loss of infectivity and (iii) HFV-based vectors should be useful for gene transfer in a wide variety of cell types.

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References


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