Persistence of selectable herpesvirus saimiri in various human
haematopoietic and epithelial cell lines

B. Simmer,1 M. Alt,2 I. Buckreus,2 S. Berthold,1 B. Fleckenstein,2 E. Platzer1 and R. Grassmann2*

1Abteilung für Hämatologie/Onkologie, Medizinische Klinik III, Friedrich-Alexander-Universität,
Krankenhausstrasse 12, W-8520 Erlangen and 2Institut für Klinische und Molekulare Virologie, Friedrich-Alexander-
Universität, Loschgestrasse 7, W-8520 Erlangen, Germany

Herpesvirus (h.) saimiri, an infectious agent of squirrel
monkeys, is capable of persisting in T lymphocytes of
various primate species. It has been used as a vector for
the functional analysis of regulatory genes in primary
human T lymphocytes. As it is not yet known whether
other cell types are capable of supporting viral
peristence, various human cell lines were investigated
using selectable h. saimiri recombinants. The lines
chosen represent cells from the epithelium and connective
tissue as well as from all haematopoietic lineages,
i.e. cells of B and T lymphoid origin as well as myeloid-
fibroblast- and carcinoma-derived cultures converted
to Geneticin or hygromycin B resistance, and harbour-
ing episomal DNA of the selectable recombinants. The
Burkitt’s lymphoma-derived cell line Raji also con-
tained simultaneously persisting episomes of the
Epstein–Barr virus. Most of the cell cultures except a
pancreatic carcinoma line and foreskin fibroblasts did
not produce infectious virus. These observations show
that a herpesvirus genome can persist episomally in a
broad range of cultured cell types. The variety of
infectable cell types and species suggests the presence
of a widely distributed and well conserved virus
receptor for h. saimiri. Thus the h. saimiri genome
could be applied more generally as a vector.

Introduction

Herpesvirus (h.) saimiri is a ubiquitous and probably
non-pathogenic agent of the New World primate Saimiri
sciureus (squirrel monkey) (for a review see Fleckenstein
& Desrosiers, 1982). It is capable of long-term persis-
tence in T lymphocytes of its natural host, and infection
of some other New World primate lymphocytes with
selected virus strains results in the immortalization of T
cells (Schirm et al., 1984; Szomolani et al., 1987) with the
CD8+ phenotype (Desrosiers et al., 1986; Kiyotaki et al.,
1986). Immortalized cells harbour persisting, circular
viral genomes in high copy number (Kaschka-Dierich et
al., 1982). Virus particles contain a linear DNA molecule
of 160 kb with the organization of a gamma-2
herpesvirus genome (Honess, 1984; Knust et al., 1983). A
central, unique region of about 112 kb (L-DNA) is
flanked by variable numbers of tandem repeats of
1-44 kb (H-DNA) (Fleckenstein et al., 1975; Bankier et
al., 1985; Stamminger et al., 1987).

The potential for long-term persistence as multicopy
episodes in T cells, and the availability of a producive
tissue culture system led to the use of h. saimiri as a
vector (Desrosiers et al., 1985; Grassmann & Flecken-
stein, 1989; Medveczky et al., 1989; Alt et al., 1991). For
example, using a non-transforming deletion variant of h.
saimiri, it was possible to demonstrate the T cell
immortalizing potential of the human T cell leukaemia
virus type 1 X region genes (Grassmann et al., 1989).

To investigate whether the h. saimiri vector could be
used in other cell types, a series of human cell lines were
infected with recombinant h. saimiri carrying dominant
selection markers. We found that far more cell lines than
expected are able to support episomal persistence of viral
genomes after infection with cell-free virus.

Methods

Virus propagation. The h. saimiri strain SIR-NEOH contains the
Geneticin (G418) resistance (neo+) marker driven by the simian virus
40 early promoter/enhancer in the context of the wild-type strain no. 11
(Grassmann & Fleckenstein, 1989). The recombinant SIR-HYG (Alt et
al., 1991) has a hygromycin B resistance (hmB+) gene under the
transcriptional control of the human cytomegalovirus immediate early
promoter/enhancer inserted in the h. saimiri strain 11-S4 (Desrosiers et
al., 1984). This strain has the 4 kb left-terminal L-DNA fragment
required for T cell immortalization deleted. Both virus strains were
propagated in an owl monkey kidney cell line (OMK-637; ATCC CRL
1556). Virus stocks were titrated by counting plaques in cultures
overlaid with medium containing 2-2% methylcellulose.

Cell culture. The suspension cultures used for infection with h.
saimiri recombinants include cell lines derived from all haematopoietic
lineages. The lymphoid lineage is represented by the T cell lines Jurkat

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ing cultures, virus particle DNA was prepared from 1 ml of

\[\text{Miyoshi et al., 1977}\]

gentamicin sulphate (100 \(\mu g/ml\)) or streptomycin/penicillin (120 \(\mu g/ml\) each).

\[\text{Grassmann & Fleckenstein, 1989}\]

for 3 to 4 weeks and periodically monitored for the appearance of

\[\text{Koefler et al., 1980}\]

splectocytes, erythrocytes and their precursors. The cell lines HEL 92.1.7

\[\text{ATCC CCL 213}\]

and Raji (ATCC CCL 86) are chosen as B cell representatives. The myeloid lineage consists of monocytes, granulocytes, and derived from acute myelogenous leukemia (Ma). The cell line BALL-1 (acute B lymphoblastic leukemia) (Miyoshi et al., 1977) and the Burkitt's lymphoma-derived lines Daudi

\[\text{ATCC CRL 1593}\]

The cell lines PANc-1 (Lieber et al., 1975) and 5637 (Gabrilove et al., 1986) are derived from human pancreatic and bladder carcinomas, respectively. Human foreskin fibroblasts (HFF), cells with limited growth potential, were derived from healthy human tissue.

\[\text{Grassmann & Fleckenstein, 1989}\]

Detection of infectious virus. To assay for the secretion of infectious virus particles, about 1 \(\times 10^6\) infected, antibiotic-resistant (neo<sup>+</sup>, hm<sup>B r</sup>) cells were added to a 25 cm<sup>2</sup> OMK-637 culture. The co-culture was kept

\[\text{Koefler et al., 1980}\]

as controls. For infection, 2 \(\times 10^6\) cells were harvested by

\[\text{Stewart, 1979}\]

on top of a vertical 1 \(\%\) agarose slab gel following the protocol of Gardella et al. (1984). Cellular DNA was separated into chromosomal, episomal and linear/degraded fractions. Gels were blotted and hybridized. Radioactive probes were obtained by nick translation of plasmids pSV2neo, pSIneo, pSi3 (Grassmann & Fleckenstein, 1989) and pORFEXHm24 (Bernard et al., 1987). Alternatively, restriction fragments containing the neo<sup>+</sup> and hm<sup>B r</sup> genes, or the

\[\text{EcoRI F}\]

Detection of viral episomes. For the detection of high Mr, superhelical DNA, resistant cells were carefully lysed on top of a vertical 1 \(\%\) agarose slab gel following the protocol of Gardella et al. (1984). Cellular DNA was separated into chromosomal, episomal and linear/degraded fractions. Gels were blotted and hybridized. Radioactive probes were prepared by nick translation from plasmid DNA containing the EcoRI I or EcoRI F fragment of h. saimiri L-DNA (Knust et al., 1983b) were isolated from these plasmids and labelled according to

\[\text{Valerius et al., 1990}\]

Human foreskin DNA analysis. Total cellular RNA was extracted by use of guanidinium rhodanidte, separated in formaldehyde gels and transferred to nitrocellulose filters as described earlier (Grassmann et al., 1989). Radioactive probes for Northern blot analyses were prepared by nick translation from plasmid DNA containing the EcoRI I or EcoRI F fragment of h. saimiri L-DNA (Knust et al., 1983). The EcoRI I fragment specifies a 1.6 kb mRNA encoding a 52K phosphoprotein. The EcoRI F fragment encodes the 28K immediate early gene product which is translated from a 1-3 kb transcript (Nicholas et al., 1988, 1990).

Analysis of interferon production. Supernatants of 5 \(\times 10^6\) cells were prepared after 24 h of culture and incubated with growing HeP-2 cells. After 24 h, infectious vesicular stomatitis virus (VSV) was added (Stewart, 1979). Viability of the cells was estimated after 48 h by their ability to reduce MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan as determined by photometric measure-

\[\text{Oez et al., 1990}\]

Surface marker analysis. Cells were incubated with monoclonal antibodies Leu 5, Leu 6, Leu 9, BL16 (Becton Dickinson), OKT3, OKT4, OKT6, OKT8, OKT26a (Ortho Diagnostics) and JOT16 (Dianova). Bound antibody was detected by fluorescein isothiocyanate (FITC)-labelled anti-mouse (Fab<sub>2</sub>), anti-lgG + M fragments (Becton

Dickinson) and flow microfluorimetry (FMF) in an electronically programmable individual cell sorter as described by Valerius et al. (1990).

Results

Conversion of human cell lines to Geneticin or hygromycin

B resistance by infection with h. saimiri recombinants

To analyse the potential of h. saimiri to infect and to persist in different types of tissue, various human cell lines were inoculated with selectable recombinants. The virus strains used (SIR-NEOH, SIR-HYG), were recombinants containing genes for neo<sup>+</sup> or hm<sup>B r</sup> phosphotransferase. Both genes are dominant selectable markers that confer resistance to the aminoglycoside antibiotics G418 and hygromycin B, respectively. The cell lines selected included fibroblasts, two carcinoma-derived cell lines and tumour lines derived from all haematopoetic lineages (Table 1). Unstimulated cells of these lines were infected with selectable h. saimiri recombinants. Wild-type virus strain 11 and its deletion mutant 11-S4 served as controls. For infection, 2 \(\times 10^5\) cells were harvested by centrifugation, resuspended in 1 ml of viral inoculum (5 \(\times 10^5\) to 1 \(\times 10^5\) p.f.u.) and incubated for 2 h at 37 °C.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Status of DNA</th>
<th>Virus production</th>
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</thead>
<tbody>
<tr>
<td>T cells</td>
<td></td>
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<tr>
<td>Jurkat</td>
<td>T-ALL</td>
<td>Episomal</td>
<td>–</td>
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<tr>
<td>CCRF-CEM</td>
<td>T-ALL</td>
<td>Episomal</td>
<td>–</td>
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<tr>
<td>Molt3</td>
<td>T-ALL</td>
<td>Episomal</td>
<td>–</td>
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<tr>
<td>B cells</td>
<td></td>
<td></td>
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<tr>
<td>BALL-1</td>
<td>B-ALL</td>
<td>Episomal</td>
<td>–</td>
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<tr>
<td>Daudi</td>
<td>BL&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Episomal</td>
<td>–</td>
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<tr>
<td>Raji</td>
<td>BL</td>
<td>Episomal</td>
<td>+</td>
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<tr>
<td>Fibroblasts</td>
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<tr>
<td>HFF</td>
<td>Human foreskin</td>
<td>Episomal/linear</td>
<td>+</td>
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<td>Epithelial cells</td>
<td></td>
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<tr>
<td>5637</td>
<td>Bladder carcinoma</td>
<td>Episomal</td>
<td>–</td>
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<tr>
<td>PANC-1</td>
<td>Bladder carcinoma</td>
<td>Episomal/linear</td>
<td>+</td>
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<td>Myeloid/erythroid cells</td>
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<tr>
<td>KS62</td>
<td>CML&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>HEL 92.1.7</td>
<td>EL&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Episomal</td>
<td>–</td>
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<tr>
<td>U937</td>
<td>HL&lt;sup&gt;+&lt;/sup&gt;</td>
<td>–</td>
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<tr>
<td>THP-1</td>
<td>AML&lt;sup&gt;+&lt;/sup&gt;</td>
<td>–</td>
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* Abbreviations: T-ALL, acute T lymphoblastic leukaemia; B-ALL, acute B lymphoblastic leukaemia; BL, Burkitt's lymphoma; CML, chronic myelogenous leukaemia; EL, erythroleukaemia; HL, histiocytic lymphoma; AML, acute monocytic leukaemia.
Cells were kept in standard medium for 48 h prior to addition of 600 to 800 µg/ml G418 or 200 µg/ml hygromycin B. Cells in control cultures had died within 2 to 4 weeks post-infection. In contrast, most of the cultures infected with the recombinants showed unaltered growth behaviour and no morphological changes were visible. With the exception of the two monocye lines, THP-1 and U-937, all cell cultures could be rendered resistant (Table 1). These experiments suggest that h. saimiri is capable of infecting and persisting in all cell lines converted to antibiotic resistance.

Phenotypic characterization of persistently infected cells

To check for production of infectious virus particles, co-cultivation experiments with the permissive OMK-637 cell line were performed. Cells from all cultures that converted to an antibiotic-resistant phenotype were checked two or three times 2 to 4 months after infection. Only Raji, HFF and PANC-1 appeared to produce virus as indicated by destruction of the OMK-637 monolayer (Table 1). Supernatants of all co-cultivation experiments were used to prepare virus particle DNA. Southern blot analysis revealed h. saimiri recombinants only in preparations derived from PANC-1, Raji and HFF cells. To monitor the antiviral effects of interferons in the co-cultivation assay, interferon production in persistently infected cell lines was examined. Supernatants of unstimulated, persistently infected lines were tested for the capacity to inhibit the c.p.e. of VSV on HeP-2 cells. None of these lines secreted detectable interferon.

To investigate the inability of cells to synthesize infectious virus, total RNA was prepared from several persistently infected cell lines. Northern blots were hybridized to nick-translated genomic h. saimiri DNA. Although RNA from lytically infected OMK-637 cells yielded numerous strongly hybridizing transcripts, RNA from persistently infected cells yielded no signal (data not shown). This indicates a general suppression of viral gene expression in these cells. Total cellular RNA of persistently infected cell lines CCRF-CEM, BALL-1, Daudi and 5637 was also analysed for two known immediate early mRNAs encoded by the EcoRI I and EcoRI F fragments of the viral genome (Fig. 1) (Knust et al., 1983b; Nicholas et al., 1988, 1990). No immediate early transcripts were detected under conditions allowing detection of less than 10 copies per cell. In contrast, approximately 10^3 molecules were present in lytically infected OMK-637 cells.

To investigate whether the presence of the viral genome affected the phenotype of T cells (CCRF-CEM and Jurkat), the expression of several surface antigens was examined. Binding of monoclonal antibodies directed against the epitopes of CD1 (Leu 6), CD2 (Leu 5), CD3 (OKT3), CD4 (OKT4), CD6 (OKT6), CD7 (Leu9), CD8 (OKT8), CD11a (IOT16), CD25 (OKT26a) and CD45 R (BL16) was analysed by FMF. No qualitative differences between infected cells and uninfected controls could be observed.

Episomal persistence of h. saimiri recombinants SIR-NEOH and SIR-HYG in resistant cell lines

After 6 weeks of growth in the presence of antibiotics, cells were tested for the persistence of viral episomes by in situ lysis gel electrophoresis (Gardella et al., 1984). This assay allows the detection of high Mr superhelical DNA after lysis of entire cells in the slots of agarose gels. To identify recombinant h. saimiri DNA, Southern blots of in situ lysis gels were hybridized with radioactive probes specific for the antibiotic resistance genes or h. saimiri sequences. All cell lines that acquired antibiotic resistance after infection with recombinant virus yielded an episomal band hybridizing to neo^-, hmB^- and h. saimiri-specific probes (Fig. 2). The HFF and PANC-1 cell lines also yielded a linear band, indicating the presence of genomic viral DNA (Gardella et al., 1984).

To establish whether selection is required for the persistence of h. saimiri episomes, G418 was removed from cultures of Jurkat, BALL-1 and CCRF-CEM cells. After 4 to 6 months of unselected growth, cells were analysed for episomal persistence by in situ lysis gel
Fig. 2. Persistence of recombinant h. saimiri in human cells. T cell lines (a), B cell lines (b), myeloid cell lines (c) and cell lines of epithelial origin (d) were infected with the h. saimiri recombinant SIR-NEOH and selected with G418. Cells (2 × 10⁶) from the resistant culture were lysed on the top of a 1% vertical agarose gel according to Gardella et al. (1984). DNA was separated by electrophoresis into chromosomal (c), episomal (e) and linear (l) DNA. Southern blots were hybridized to a SIR-NEOH-specific probe (b) or to an EBV-specific probe (a). Lanes 2, uninfected controls.

Fig. 3. Double persistence of h. saimiri SIR-NEOH and EBV in Raji cells. The Raji cell line was infected with SIR-NEOH (lanes 2) and treated with G418. Cells (2 × 10⁶) from the resistant culture were lysed on the top of a 1% agarose gel. DNA was separated by electrophoresis into chromosomal (c), episomal (e) and linear (l) DNA. Southern blots were hybridized to a SIR-NEOH-specific probe (b) or to an EBV-specific probe (a). Lanes 2, uninfected controls.

electrophoresis. All cell lines were positive for high Mr episomal DNA hybridizing to the neo' gene. The amount of DNA detected, however, was five- to tenfold less than that from selected controls. This in summary demonstrates the capability of h. saimiri recombinants to infect and to persist in various human cell types.

Simultaneous episomal persistence of h. saimiri and Epstein–Barr virus (EBV)

The Raji cell line contains episomes of EBV, another gammaherpesvirus (Roizman, 1982). The relationship of EBV to h. saimiri is reflected by sequence similarities in many viral proteins (Cameron et al., 1987; Gompels et al., 1988; Nicholas et al., 1988; Albrecht & Fleckenstein, 1990). To examine whether a second gammaherpesvirus genome would interfere with EBV persistence, Raji cells were also checked for persisting EBV DNA. Hybridization of blotted in situ lysis gels with cosmid clone cMSalB (Polack et al., 1984) revealed strongly hybridizing bands of identical intensity in cells infected with h. saimiri and uninfected controls (Fig. 3). This experiment indicates co-persistence of EBV and h. saimiri in the same cell culture.
 Persistence of h. saimiri in human cell lines

Discussion

The capacity of various human haematopoietic and monolayer cells to support persistent infection of h. saimiri was investigated using selectable recombinants. These virus strains were previously shown to be capable of persisting in a human T cell line (Grassmann & Fleckenstein, 1989; Alt et al., 1991). Here we demonstrate viral persistence in human fibroblast, epithelial, myeloid and B lymphoid cells and in two other T cell lines. This broad range of infectable cell types suggests a widely distributed virus receptor. The observation that rabbit (Medveczky et al., 1989) and rodent cells (M. Alt, B. Fleckenstein & R. Grassmann, unpublished data) are also infectable suggests that this receptor is well conserved.

Most of the cell lines did not produce infectious viral particles or detectable amounts of interferon which could interfere with the co-cultivation assay. Co-cultivation experiments with the h. saimiri-inoculated cell lines Raji, HFF and PANC-1, however, revealed the presence of infectious virus. This is in agreement with the appearance of a prominent band of linear virus DNA in in situ lysis gels of PANC-1 and HFF cells. No corresponding band was visible when Raji cells were investigated. This points to rather low rates of viral replication and may precede the total disappearance of virus production.

All cell lines which converted to antibiotic resistance after infection with the recombinants contained viral genomes as high Mr episomal DNA. This indicates that all functions required for episomal replication are present in these cell types and therefore such cells should be suitable for the detection and analysis of the h. saimiri origin of persistent replication (oriP). The Raji cell culture, which was rendered antibiotic-resistant after infection with the h. saimiri recombinant SIR-NEOH, contained episomal DNA of both herpesviruses. As the presence of the neo' gene is required for G418 resistance, this indicates double persistence of EBV and h. saimiri within the same cell.

Viral episomes could be demonstrated in cells long after release from selective pressure (about 150 doublings). This suggests that there is no major counter-selection against h. saimiri persistence. The five- to 10-fold reduction of copy number observed may be explained by a selective growth advantage of cells containing an increased gene dosage of resistance markers under selective conditions.

The lack of counter-selection and the absence of phenotypic changes in T lymphocytes favour a model of h. saimiri persistence with strongly suppressed viral gene activity. This could be due to a failure to support viral transcription especially at the immediate early phase of the replication cycle. Northern blots of total cellular RNA derived from several cell lines failed to reveal the two immediate early transcripts (Nicholas et al., 1988, 1990) but could demonstrate neo' mRNA. Since the expression of herpesvirus immediate early genes is a prerequisite for the synthesis of late mRNA encoding structural proteins (Stinski, 1983; Wagner, 1985), this observation could explain the absence of infectious virus production.

Permanent expression of the regulatory EBNA-1 protein is required for episomal persistence of the EBV genome (Yates et al., 1985). Although the mechanism of h. saimiri episomal maintenance is still unclear, there is no evidence for the requirement of an EBNA-1-related function. Nuclear antigens could not be detected in monkey T cell lines harbouring circular viral genomes (Fleckenstein & Desrosiers, 1982) and the sequence of the virus does not indicate an open reading frame with significant homology (B. Biesinger, J.-C. Albrecht & B. Fleckenstein, unpublished data). Analysis of viral mRNA expression in a tumour cell line containing h. saimiri episomal DNA revealed the transcription of a single gene (Knust et al., 1983a) encoding thymidylate synthetase (Bodemer et al., 1986; Honess et al., 1986).

The h. saimiri vector system uses infectious virus particles prepared from a fully permissive cell line for the transduction of heterologous genes in cell lines and in primary cells. After infection, the recombinant viral genomes persist as multicopy episomes. These features, and the wide range of infectable cells reported here, suggest a more general applicability of this vector for human cells.

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