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 persistence, 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 harbouring episomal DNA of the selectable recombinants. The Burkitt’s lymphoma-derived cell line Raji also contained 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 episomal 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 persistence 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., 1983b). 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; Stammingier et al., 1987).

The potential for long-term persistence as multicopy episomes in T cells, and the availability of a productive tissue culture system led to the use of h. saimiri as a vector (Desrosiers et al., 1985; Grassmann & Fleckenstein, 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
(Schneider et al., 1977), Molt3 (ATCC CRL 1552) and CCRF-CEM
(ATCC CCL 119) which were established from acute T lymphoblastic
leukaemia. The cell line BALL-1 (acute B lymphoblastic leukaemia
(Miyoshi et al., 1977) and the Burkitt's lymphoma-derived lines Daudi
(ATCC CCL 213) and Raji (ATCC CCL 86) were chosen as B cell
representatives. The myeloid lineage consists of monocytes, granulo-
cytes, erythrocytes and their precursors. The cell lines HEL 92.1.7
(ATCC TIB 180) and K 562 (ATCC CCL 243) are of myeloid origin, and
are derived from erythroleukaemia and chronic myelogenous leuka-
emia (Koeffler et al., 1980). The monocyte section of the myeloid lineage
is represented by the cell lines THP-1 (ATCC TIB 202) and U-937
(ATCC CRL 1593). The cell line PAN-C-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.

All suspension cultures and cell line 5637 were kept in RPMI 1640
medium, cell lines OMK-637 and HFF in MEM, and the pancreas
 carcinoma-derived cells (PANC-1) in Dulbecco’s MEM. All media
were supplemented with 10% foetal calf serum, glutamine (2 mM)
and gentamicin sulphate (100 μg/ml) or streptomycin/penicillin (120 μg/ml
each).

Detection of infectious virus. To assay for the secretion of infectious
virus particles, about 1 x 10^6 infected, antibiotic-resistant (neo^r, hmB^r)
cells were added to a 25 cm² OMK-637 culture. The co-culture was kept
for 3 to 4 weeks and periodically monitored for the appearance of
cytopathic changes. To demonstrate replicating h. saimiri in degenerat-
ing cultures, virus particle DNA was prepared from 1 ml of supernatant (Grassmann & Fleckenstein, 1989) and hybridized to a
specific probe.

Detection of viral episomes. For the detection of high M, superhelical
DNA, resistant cells were carefully lysed on top of a vertical 1.3%
agarose slab gel following the protocol of Gardella et al. (1984). Cellular
DNA was separated into chromosomal, episomal and linear/degraded
fractions. Gels were botted 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^r
and hmB^r genes, or the Kpnl E fragment of h. saimiri (Knust
et al., 1989). Radioactive probes for Northern blot analyses were prepared by
nick translation from plasmid DNA containing the
\[ E \text{ fragment of } h. \text{saimiri L-DNA} \] (Knust et al., 1983b) were isolated from these plasmids and labelled according to

RNA analysis. Total cellular RNA was extracted by use of
guanidinium rhodanide, separated in formaldehyde gels and trans-
ferred 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 (Knust et al., 1983b). The EcoRI I fragment
specifies a 1.6 kb mRNA encoding a 52K phosphoprotein. The EcoRI
F fragment encodes the 28K immediate early gene protein which is
translated from a 1:3 kb transcript (Nicholas et al., 1988, 1990).

Analysis of interferon production. Supernatants of 5 x 10^6 cells were
prepared after 72 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-diphenyltetra-
zolium bromide] to formazan as determined by photometric measure-
ment (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), anti-iGg + M fragments (Becton
Dickinson) and flow microfluorimetry (FMF) in an electronically
programmable individual cell sorter as described by Valerius et al.

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 recom-
binants containing genes for neo^r or hmB^r phosphotrans-
ferase. Both genes are dominant selectable markers that
confer resistance to the aminoglycoside antibiotics G418
and hygromycin B, respectively. The cell types 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 11-54 served
as controls. For infection, 2 x 10^6 cells were harvested by
centrifugation, resuspended in 1 ml of viral inoculum
(5 x 10^5 to 1 x 10^6 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>
</tr>
</thead>
<tbody>
<tr>
<td>T cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jurkat</td>
<td>T-ALL*</td>
<td>Episomal</td>
<td>–</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>T-ALL</td>
<td>Episomal</td>
<td>–</td>
</tr>
<tr>
<td>Molt3</td>
<td>T-ALL</td>
<td>Episomal</td>
<td>–</td>
</tr>
<tr>
<td>B cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BALL-1</td>
<td>B-ALL*</td>
<td>Episomal</td>
<td>–</td>
</tr>
<tr>
<td>Daudi</td>
<td>BL*</td>
<td>Episomal</td>
<td>–</td>
</tr>
<tr>
<td>Raji</td>
<td>BL</td>
<td>Episomal</td>
<td>+</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HFF</td>
<td>Human foreskin</td>
<td>Episomal/ linear</td>
<td>+</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>5637</td>
<td>Bladder carcinoma</td>
<td>Episomal</td>
</tr>
<tr>
<td>PANC-1</td>
<td>Pancreatic carcinoma</td>
<td>Episomal/ linear</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Myeloid/erythroid cells</th>
<th></th>
<th>Episomal/ linear</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562</td>
<td>CML*</td>
<td>Episomal</td>
</tr>
<tr>
<td>HEL 92.1.7</td>
<td>EL*</td>
<td>Episomal</td>
</tr>
<tr>
<td>U937</td>
<td>HL*</td>
<td>–</td>
</tr>
<tr>
<td>THP-1</td>
<td>AML*</td>
<td>–</td>
</tr>
</tbody>
</table>

* 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 monocytic cell 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^r-, hmB^r- 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
**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 cMasB (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 *M*, 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 persistance 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.

This work was supported by the Deutsche Forschungsgemeinschaft, Forscherguppe: 'DNA-Viren des hämatopoetischen Systems'.

References


**Cameron, K. R., Staminger, T., Craxton, M., Bodemer, W., Honess, R. W. & Fleckenstein, B.** (1987). The 160,000-M, virion protein encoded at the right end of the herpesvirus saimiri genome is homologous to the 140,000-M, membrane antigen encoded at the left end of the Epstein-Barr virus genome. *Journal of Virology* 61, 2063–2070.