Generation of Herpes Virus Saimiri-Transformed T-Cell Lines from Macaques Is Restricted by Reactivation of Simian Spuma Viruses

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Herpes virus saimiri (HVS) transforms human T-cells in vitro to stable growth. These T-cell lines retain their immunological characteristics of the parent cells and do not release infectious virus. Recently, lymphocytes of Old World monkeys were efficiently transformed by HVS. In parallel to these studies we initiated transformation experiments by infecting peripheral blood cell cultures of 45 monkeys, 35 rhesus and 10 cynomolgus macaques. In only three cases, we obtained transformed T-cell lines. The transformed T-cells were largely double-positive for CD4 and CD8. They responded with increased proliferation to mitogenic or IL-2 stimulation and transcribed mRNA for IL-2, IL-4, and IL-10. However, most initiated T-cell cultures from macaques developed giant cells. The cytopathic agent was identified as simian foamy virus (SFV) as confirmed by PCR, immunofluorescence, and coculture experiments. Treatment of the T-cell cultures with AZT- and SFV-specific sera did only shortly prolong the life-span of the cultures. Therefore, the reactivation of SFV caused remarkable difficulties in the establishment of macaque T-cell lines by HVS. This seems to be a general problem since most animals from several breeding colonies are SFV-positive.

INTRODUCTION

Herpes virus saimiri (HVS) is the prototype of γ2-herpes viruses or rhadino viruses (Roizman et al., 1992) and is apathogenic in its natural host, the squirrel monkey (Saimiri sciureus). In contrast to this benign behavior in its natural host, HVS has a high oncogenic potential in numerous other New World primates and causes acute lymphoproliferative syndromes, leukemias, and T-cell lymphomas (Fleckenstein and Desrosiers, 1982). In addition, HVS is able to transform T-lymphocytes of nonhuman primates in culture (Schirm et al., 1984). Furthermore, human primary T-lymphocytes are readily transformed by wild-type subgroup C strains of HVS to continuously proliferating T-cell lines (Biesinger et al., 1992). These transformed human T-cell lines retain essential properties of their parent cells and do not release infectious virus (Mittrücker et al., 1992; Bröker et al., 1993; De Carli et al., 1993; Del Prete et al., 1994). Besides, HVS-transformed human T-cells are a permissive system for immunodeficiency viruses (Nick et al., 1993). Recently, Akari et al. (1996) reported that HVS efficiently transformed lymphocytes of Old World monkeys, rhesus (Mm, Macaca mulatta), and cynomolgus monkeys (Mf, Macaca fascicularis), resulting in continuously proliferating T-cell lines and that these cell lines were highly susceptible to simian immunodeficiency virus (SIV) infection. In parallel to these studies, we used strain C 488 of HVS grown on epithelial owl monkey kidney (OMK) cells to infect macaque T-cells (Desrosiers and Falk, 1982). In total, 45 cultures of peripheral blood mononuclear cells (PBMC) from 35 rhesus and 10 cynomolgus macaques were initiated. Only 3 of the 45 cultures became successfully transformed T-cell lines. Most of the initiated cell lines developed syncytia due to simian foamy virus (SFV) reactivation. Thus, the SFV infection of macaques is a general problem for generation of HVS-transformed T-cell lines.

MATERIALS AND METHODS

Cell culture, Transformation of monkey PBMC, and detection of viral DNA

Peripheral blood mononuclear cells from the macaques were obtained from citrated blood by Ficollidiatrizoate centrifugation (Histopaque, Sigma, Germany). Cells (1 × 10⁶ per ml) were seeded in a medium composed of 45% RPMI 1640, 45% CG medium (Vitromex, Vilshofen, Germany), 10% heat-inactivated fetal calf serum (FCS) supplemented with 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. The PBMC were stimulated with 1% (v/v) phytohemagglutinin (PHA, Murex Diagnostics Limited, England) or 25 U/ml recombinant in-
terleukin 2 (IL-2) (GBF, Braunschweig, Germany) for 1 day. Subsequently infectious supernatant (10% (v/v)) from lytically infected OMK cells were added. The infectious supernatant from OMK cells was obtained essentially as described (Fickenscher and Fleckenstein, 1994). The culture medium was changed twice a week and once IL-2 was added.

The detection of viral DNA in HVS-transformed cells was performed by the method of Gardella et al. (1984) with a 32P-labeled Stp C fragment of HVS strain C 488 (Biesinger et al., 1990; Fickenscher and Fleckenstein, 1994).

We conducted an HVS-specific polymerase chain-reaction (PCR) for the gene tip and the oncogene stp C 488; both are derived from a bicistronic mRNA encoded in the left terminal part of the unique DNA of HVS (Biesinger et al., 1995). The PCR protocol was established by Dr. A. Ensser (Erlangen, Germany). For the PCR, template DNA was prepared from HVS-transformed T-cell lines. About 1 μg of DNA was added to each PCR mixture containing 1.5 mM Mg2+, 0.1% Triton-X 100, 200 mmol of each deoxy nucleoside triphosphate (dNTP), 25 pmol of each oligonucleotide primer, and 1 U Taq polymerase (Technomara, Fernwald, Germany). The tip and stp C 488 sequences were detected using the following primers: tip Primer 1, 5′ CTITTTATTACACACCATGCACAAG 3′; tip Primer 2, 5′ GTGAAATACAAACGACCAAAAC 3′; stp C 488 Primer 1, 5′ CTATAATGCTTGGCTTCTTAG 3′; stp C 488 Primer 2, 5′ CACCAACAGCTTCTAGAAGGAGC 3′. The amplification reaction for both HVS sequences was performed under the following cycle profile: denaturation at 94° for 1 min, annealing at 60° for 30 sec, and extension for 1 min at 72°. The PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide.

Phenotypical characterization of HVS-transformed T-cell lines

To determine expression of cell surface antigens transformed T-cells were stained with an anti-CD4 monoclonal antibody (MAb; OKT4; Ortho Diagnostics); anti-CD8 MAb (Leu 2a; Becton – Dickinson) and anti-CD2 MAb (Leu5b; Becton – Dickinson). The MAb were either coupled to FITC or phycoerythrin and diluted according to the manufacturer’s instructions. After staining, samples were analyzed by flow cytometry on an Epics Profile (Coulter).

The cytokine profile of HVS-transformed T-cell lines were analyzed by RT-PCR as described by Dittmer et al. (1996).

To study the influence of exogenous stimulation the transformed T-cells were seeded in microtiter plates (1 × 105 cells/well) in 100 μl CG medium supplemented with 1% human AB serum. Cells were stimulated with 1% Concanavalin A, 1% PHA, or 25 U/ml IL-2 per well and incubated for 7 days at 37°. For the last 6 hr of culture 0.5 μCi [3H]thymidine was added per well. The cells were harvested and the incorporated radioactivity was determined in a β-counter.

Detection of SFV

SFV antigens were detected by indirect immunofluorescence (Voss et al., 1992). Briefly, 1 × 106 cells were removed from T-cell cultures and washed once in phosphate-buffered saline (PBS, 2 mM KH2PO4; 9 mM Na2HPO4, 180 mM NaCl, pH 7.2). Cells were resuspended in 100 μl PBS and aliquots of 10 μl were pipetted onto glass slides. The slides were dried for 1 hr at RT. The cells were fixed for 15 min at −20° in methanol and washed in PBS. Sera were diluted 1:10 in PBS and incubated with the cells for 30 min at 37° in a humidified atmosphere. After washing with PBS a FITC-labeled goat-anti-human IgG antiserum (Meda, Germany) diluted 1:20 was incubated with the cells for 20 min. The slides were washed once again and covered with 20% (v/v) glycerin in PBS prior to examination with a fluorescence microscope (Zeiss, Germany). SFV antigen was examined with positive reference sera from infected macaques.

The presence of proviral SFV in the T-cell lines was confirmed by a conventional DNA PCR which was originally described as a nested PCR (Schweizer et al., 1994, 1995). Denaturated DNA (500 ng to 1 μg) was amplified in an 50-μl reaction containing 1.5 mM Mg2+, 0.1% Triton X-100, 200 mmol of each dNTP, 25 pmol of each primer, and 1 U Taq polymerase. Foamy virus pol sequences were detected using the following primers (Schweizer et al., 1994, 1995): Primer 1, 5′ CCTGGATGCAGAGTTGG min at 72°. The amplification reaction was performed under the following conditions: 1 min of denaturation at 94°, 1 min of annealing, 2 min of extension at 72°. The annealing temperature was 38° in the 2 initial cycles and 55° in the subsequent 28 cycles. Amplification products were analyzed in 1.5% agarose gels and visualized by ethidium bromide staining.

<table>
<thead>
<tr>
<th>Transforming virus</th>
<th>Total</th>
<th>Mm</th>
<th>Mf</th>
<th>Humans</th>
</tr>
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<tr>
<td>HVS C 488</td>
<td>45/0</td>
<td>35/3</td>
<td>10/0</td>
<td>90–100%</td>
</tr>
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a Mm, Macaca mulatta; Mf, Macaca fascicularis.
b Number of initiated cultures/number of successfully transformed cell lines.
c Number of initiated cultures without HVS/number of spontaneously transformed cell lines.
FIG. 1. Syncytia formation in a herpes virus saimiri (HVS)-transformed T-cell line of rhesus monkey Mm 7086 (A). (B) Shows a control culture without giant cell formation. Cultures were analyzed by light microscopy (Zeiss, Germany) ×100. Syncytia are indicated by an arrow.
To detect infectious SFV coculture experiments with transformed T-cells and human embryonal lung fibroblasts (HEL S37) were performed (Voss et al., 1992). HEL S37 were grown to a confluent monolayer. Subsequently, the cells were cocultivated with $1 \times 10^5$ transformed T-cells or with SFV-free cells as control. Formation of syncytia was microscopically examined.

To suppress SFV replication the T-cells were split and incubated with different 3'-azido-3'-deoxythymidine (AZT) concentrations ranging from 0.1 to 5 $\mu$mol per milliliter added at different intervals (daily, once per week, 3x per week) (Voss et al., 1992). A combination therapy with AZT and 3% sera from SFV-infected rhesus monkeys (Neumann-Haefelin et al., 1986) was also performed to prevent the spreading of infectious virus by neutralizing antibodies.

**RESULTS**

In order to transform macaque T-cells with HVS, a total of 45 cultures of PBMC from 35 rhesus and 10 cynomolgus macaques were initiated. However, only 3 of the 45 cultures became successfully transformed T-cell lines (Table 1). In these three cell lines dividing cells were not visible before 2 months of culture. Successful transformation with HVS C 488 was only achieved when the PBMC were prestimulated for 24 hr with PHA.

In contrast, after 2-3 weeks in culture syncytia were observed in all other initiated cultures. The number of syncytia cells increased in these cultures and the cells died within 2 months. All cultures with syncytia had been initiated from PBMC of animals seropositive for SFV as shown by indirect immunofluorescence. Only two cultures (Mm 7084 and Mm 7205) were derived from SFV-seronegative rhesus monkeys and did not show syncytia, whereas the culture of Mm 7086 permanently demonstrated a low number of giant cell formation (Fig. 1). The transformed rhesus T-cells showed continuous growth for more than 6 months and contained episomal HVS-DNA as detected in Gardella gels. To confirm the results of the Gardella gel electrophoresis we conducted an HVS-specific PCR. In all three T-cell lines the amplification products of the oncogene stp C 488 (348 bp) and tip (843 bp) were detectable (data not shown). The transformed T-cells showed the phenotype of activated T-cells. They expressed the T-cell-specific marker CD2, but approximately 50% of the T-cells were double positive for CD4 and CD8 (data not shown). They responded with increased proliferation after mitogenic or IL-2 stimulation in a proliferation assay. In addition, we measured the mRNA synthesis of three cytokine genes in a cytokine-specific RT-PCR. The expression of IL-2, IL-4, and IL-10 mRNA was detectable in all three transformed T-cell lines (data not shown). These results showed that the transformed T-cells retain essential properties of their parental cells, including cytokine gene transcription and proliferative responses. However, syncytia were observed in the cultures of the SFV-seronegative macaques (Mm 7084, Mm 7205) after thawing of frozen cultures. Therefore, we established a modified foamy-specific macaques protocol to detect proviral SFV-DNA in T-cells. All three generated T-cell lines were positive for foamy provirus by PCR amplification of the pol gene (Fig. 2A). Moreover, the PBMC of Mm 7084 prior to transformation with HVS were positive for foamy provirus by PCR amplification as well as the transformed T-cell line of this monkey prior to freezing (Fig. 2B). These results implied that SFV-seronegative macaques might be latently infected with SFV and that the virus can be reactivated in vitro.

In addition, the cultures with giant cell formation

![Fig. 2. Polymerase chain reaction (PCR) amplification of the simian foamy virus (SFV) pol gene in rhesus monkey T-cell lines transformed by strain 488 of herpes virus saimiri (HVS) group C. 10$^{-5}$ $\mu$l of each amplification product was analyzed on a 1.5% agarose gel and visualized by ethidium bromide staining. (A) The DNA from a SFV-negative B-cell line (lane 1) and from SFV-infected Vero L-cells (lane 2) were used as negative or positive control. In lanes 3, 4, and 5 DNA from the HVS-transformed T-cell lines Mm 7205 (lane 3), Mm 7086 (lane 4), and Mm 7084 (lane 5) was analyzed. Lane 6 shows the negative control without DNA and (M) represents the DNA marker. (B) Lane 1 and lane 2 shows the same positive and negative control as used in (A). In lane 3 DNA from the HVS C 488 virus stock was analyzed. Lane 4 contained the DNA from PBMC of Mm 7084 prior to transformation with HVS. In lane 5 DNA from the HVS-transformed T-cell line Mm 7084 prior freezing was analyzed. Lane 6 shows the control without DNA and (M) represents the DNA marker. Numbers at the left indicate DNA size in base pairs (bp). The gel was documented by using the Gelprint 2000i gel documentation system and ONE-D-Scan software (MWG Biotech, Ebersberg, Germany).](image-url)
FIG. 3. Simian foamy virus (SFV) antigens in HVS-transformed T-cells detected by indirect immunofluorescence. The T-cell lines were fixed with cold methanol and incubated with SFV-positive (A) or negative (B) reference sera, followed by staining with FITC-conjugated goat anti-human IgG antibodies. Immunofluorescence was detected with a Zeiss fluorescence microscope.

showed the presence of SFV antigens determined by indirect immunofluorescence (Fig. 3). To detect infectious SFV, human embryonal fibroblasts (HEL S 37) were cocultivated with T-cell lines positive for SFV-induced syncytia. After 48 hr of coculture with SFV-positive T-cell lines Hel S 37 cells exhibited multinucleated syncytia, indicating the release of infectious SFV by these T-cell lines. Voss et al. (1992) described an inhibition of SFV-induced giant cell formation in B-cell lines treated with AZT. We also attempted to suppress SFV replication in HVS-transformed T-cell lines with AZT. These treatment experiments demonstrated that a concentration between 1 and 5 μmol AZT per milliliter were cytotoxic to T-cells. Therefore, only low concentration of AZT could be used for the treatment of T-cell lines. With these AZT-concentrations we did not observe any inhibition of giant cell formation in the treated T-cell cultures and also observed no significant prolongation of their life span. Recently, Schweizer et al. (1993) reported that cell cultures latently infected with SFV-3 were established by suppressing lytic infection in Vero cells with AZT- and SFV-specific antibodies. In contrast to their findings, we observed only
a delayed increase of giant cell formation after the treatment of transformed T-cell lines with AZT (1 μg/ml) and 3% serum from SFV-positive macaques. However, the SFV reactivation led to the ultimate loss of the T-cell lines.

**DISCUSSION**

Similar to the experimental approach reported by Akari et al. (1996), we have transformed T-cells from Old World Monkeys with subgroup C strain 488 of HVS. We were able to generate three T-cell lines from HVS-infected PBMC cultures of 45 macaques. As it has been described for transformed human T-cells (Biesinger et al., 1992), these three transformed T-cell lines contained HVS genomes exclusively in an episomal state. The transformed T-cells retained properties of conventional T-cells such as cytokine production and proliferative responses even after continuous culture for several months. Similar to transformed human T-cells (Biesinger et al., 1992) the rhesus T-cell lines showed the phenotype of activated T-cells. Remarkably, transformed rhesus T-cells were largely double-positive for CD4 and CD8. This double expression of CD4 and CD8 on rhesus T-cells could be due to the expansion of a lymphocyte subpopulation of an early or intermediate stage in differentiation or resulted from reexpression of CD4 or CD8 on single positive cells.

Unexpectedly, the majority of cultures exhibited syncytia 2–3 weeks after initiation. Many of these cultures could not be maintained longer than a month. Foamy virus infection of these cells was the most probable cause of cell death. The giant cells were shown to contain SFV antigen and infectious virus was transmitted to human embryonal fibroblast. In addition, we detected SFV provirus by PCR in the T-cell lines. We attempt to suppress giant cell formation by treatment of cells with AZT (Voss et al., 1992) or AZT and sera from SFV-positive macaques (Schweizer et al., 1993). However, giant cell formation could not be inhibited and life-span of the treated cultures was only shortly prolonged. Obviously, the replication of SFV causes remarkable difficulties for the establishment of simian T-cell lines by HVS similar to those reported for transformation of B-cells by herpes virus papio (Voss et al., 1992). These results confirm Lear et al. (1996) who have shown that B- and T-lymphocytes of monkeys are the major reservoir for SFV, whereas human lymphocytes are usually spumavirus-negative. Unfortunately, over 90% of the monkeys in our colony are SFV-seropositive. A similar seroprevalence was found in animals imported from the Cayo Santiago stock (Puerto Rico) or from the Laboratory Animal Breeders and Services (Yemassee, South Carolina). Furthermore, some of the SFV-seronegative macaques harbor latent SFV as shown by PCR and SFV can be reactivated in cells cultured from these animals. Therefore, simian T-cell lines can only be established from rarely found SFV-free macaques. To circumvent this problem we established T-cell clones from rhesus and cynomolgus monkeys by limiting dilution techniques. SFV-free T-cell clones can then be selected by PCR and thereafter transformed with HVS C 488. Despite the problems of establishing HVS transformed T-cell lines from macaques the immortalization with HVS is a powerful tool to study T-cell biology and antigen specificity in primates. In addition, transformed T-cells can be used as a permissive system for immunodeficiency viruses (Akari et al., 1996; Nick et al., 1993).

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virus (SIV) did not protect them against the consequences of a high-dose SIV challenge. J. Gen. Virol. 76, 1307–1315.


