Human herpesvirus-8 (Kaposi’s sarcoma-associated virus) ORF50 increases in vitro cell susceptibility to human immunodeficiency virus type 1 infection

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INTRODUCTION

Human herpesvirus-8 (HHV-8) is the most recently discovered member of the family Herpesviridae that infects humans (Chang et al., 1994). Several evidences support the viral causative role in the pathogenesis of Kaposi’s sarcoma (KS) (Schultz, 2000), the most common neoplasm in human immunodeficiency virus (HIV)-infected patients. HHV-8 is associated also with two other neoplastic diseases, primary effusion lymphoma (PEL) and multicentric Castleman’s disease, rare lymphatic proliferations that affect HIV-infected patients (Cesarman et al., 1995; Grandadam et al., 1997). The incidence of HHV-8 infection in the healthy population is characterized by a significant geographical variation and is higher in areas where KS is more common. Accordingly, the seroprevalence for HHV-8 ranges from 0 to 5 % in northern Europe and America (with low incidence of KS) to 30 % in the southern Mediterranean areas (where classic KS may affect the elderly Caucasian population) and reaches over 50 % in parts of tropical Africa (endemic for aggressive forms of KS) (Gao et al., 1996; Kedes et al., 1996; Lennette et al., 1996; Whitby et al., 1998). The incidence of HHV-8 infection is significantly higher in HIV-infected patients, even in areas where the virus is rarely detected in the healthy population, as shown by a HHV-8 seropositivity of 30–40 % in HIV-positive homosexual men in the USA and northern Europe (Fitzpatrick et al., 1999; Gao et al., 1996; Kedes et al., 1996).

HHV-8 DNA is detected not only in neoplastic lesions but also in the peripheral blood of KS patients, mainly in CD19+ and CD22+ B cells (Monini et al., 1999; Whitby et al., 1995), and in endothelial lineage-derived spindle cells and monocytes within the KS lesion (Staskus et al., 1997; Blasig et al., 1997). It was also shown recently that HHV-8 can infect primary human keratinocytes and dermal microvascular endothelial cells, inducing alterations of growth properties that include the development of a spindle-like phenotype (Cerimele et al., 2001; Ciufo et al., 2001).

Development of HHV-8-associated tumours is closely related to the replicative activity of the virus. In fact, a critical step in HHV-8 oncogenesis is represented by the activation of virus infection, mediated by the switch between latent and productive phases (Ensoli et al., 2001; Gruffat et al., 2000). ORF50 and ORF57 play a crucial role in the regulation of virus replication and reactivation. ORF50 is homologous to Epstein–Barr virus lytic switch protein BRLF1 (also named Rta) and encodes an immediate-early protein with a strong transactivating effect (Gradoville et al., 1999).
The high prevalence of HIV/HHV-8 co-infection in HIV-positive individuals suggests the possibility that the two viruses can interact. Current evidence shows that HIV plays an important role in the pathogenesis of AIDS-related KS, even though retrovirus infection is not an absolute requirement for the development of KS (Foreman, 2001). It is postulated that HIV might promote the initiation and progression of KS by the biological effects of the Tat protein and by promoting cytokine production (Gallo, 1998). Published studies indicate that HIV can interact directly with HHV-8. In fact, HIV infection stimulates HHV-8 replication, both in co-infected cells and in adjacent cells (Varthakavi et al., 1999), and HIV-1 Tat induces the lytic cycle replication of HHV-8 (Harrington et al., 1997). Recent data suggest also that HHV-8 can have direct effects on HIV infection. Hyun et al. (2001) reported that the latency-associated nuclear antigen of HHV-8 activates the long terminal repeat (LTR) of HIV and stimulates expression of HIV p24. We have shown recently that ORF50 interacts synergistically with HIV-1 tat at a post-transcriptional level, inducing a 10-fold enhancement of HIV-1 LTR transcription. Therefore, the presence of ORF50 might render very small amounts of Tat transcriptionally active (Caselli et al., 2001).

With the aim of determining whether the molecular activation of HHV-8 on HIV results in significant biological interactions, we studied the effects of ORF50 and ORF57 on HIV replication and production. Cell lines, either permissive or non-permissive to HIV, were transfected with recombinant plasmids containing HHV-8 ORF50 or ORF57 and infected with HIV. Virus replication was studied by PCR, RT-PCR, and titration. Lymphoid T and B cell lines were grown in RPMI medium (Gibco) supplemented with 10% inactivated foetal bovine serum (FBS), 2 mM L-glutamine, 100 U penicillin ml\(^{-1}\) and 100 μg streptomycin ml\(^{-1}\). Glioblastoma A172 cells were cultured in DMEM medium with 10% FBS, 2 mM L-glutamine, 100 U penicillin ml\(^{-1}\) and 100 μg streptomycin ml\(^{-1}\). All cells were cultured at 37°C in the presence of 5% CO\(_2\).

**Cell transfections and selection of stable clones.** Each cell line was transfected to obtain permanent clones stably expressing the mature transcripts of ORF50 or ORF57 (plasmids pCR-50sp and pCR-57sp, respectively). Cells lines were passaged 24 h before transfection to obtain optimal cellular density. T and B lymphoid cells were suspended in serum-free medium (10\(^7\) cells in 1 or 0.5 ml, respectively) and transfeected by electroporation (960 μF, 250 mV) with 10 μg plasmid DNA using a GenePulser II (Bio-Rad). At 24 h after transfection, cells were seeded in 96-well plates in the presence of 400 μg neomycin (G418) ml\(^{-1}\) (Roche) for selection of stable clones.

A172 cells were seeded in 6 cm diameter plates (5 × 10\(^5\) cells per plate) and transfected with 5 μg plasmid DNA by calcium phosphate transfections. For selection of stable clones, cells were passaged in 10 cm diameter plates in the presence of 400 μg neomycin ml\(^{-1}\). Transformed foci were evident after about 14 days.

Both lymphoid and neuronal cell clones were harvested, propagated and characterized by PCR and RT-PCR for the presence and transcription of the 50sp and 57sp genes, using primers and PCR conditions described previously (Caselli et al., 2001). Clones with the highest levels of specific transcripts were selected for subsequent experiments.

**HIV-1 infection.** Virus stocks were produced infecting H9 T cells with HIV-1 strain IIIB. Virus was collected from cell supernatant 14 days post-infection (p.i.). Virus titres were determined by the Reed and Muench dilution method on C8166 T cells infected with serial dilutions of virus supernatant, in quadruplicate. The titre of the virus stock used in all assays was 2 × 10\(^{6}\) CCID\(_{50}\) ml\(^{-1}\).

Jurkat cells, stably transformed with ORF50 or ORF57, were infected with an m.o.i. of 1:10000 and 1:100000. Briefly, 10\(^6\) cells ml\(^{-1}\) were incubated with 10\(^5\) or 10\(^7\) CCID\(_{50}\) ml\(^{-1}\) HIV-1 for 24 h at 37°C in the presence of 5% CO\(_2\). After infection, cells were centrifuged, washed twice with PBS, suspended in RPMI complete medium and seeded in sterile flasks at the density of 5 × 10\(^5\) cells ml\(^{-1}\). Samples consisting of 10 ml of cell suspension were collected every 4 days (0, 4, 8, 12, 16, 20, 24 and 28 days p.i.). Cells were collected by centrifugation, washed with PBS, divided into two aliquots for DNA and RNA analyses, frozen in liquid nitrogen and kept at −80°C until use. Aliquots of supernatant were frozen at −80°C for virus rescue and p24 analyses.

BC-3 cells were infected with different m.o.i., from 1:10 to 1:10000. Cell samples were collected as described, at 0, 3, 7, 14, 21 and 28 days p.i.

**METHODS**

**Recombinant plasmid DNAs.** Spliced forms of ORF50 and ORF57 were cloned in the expression vector pCR3.1-Uni (Invitrogen), as described previously (Caselli et al., 2001). Briefly, spliced genes were obtained from TPA-activated BCBL-1 cells by specific retrotranscription of poly(A)\(^{+}\) RNA followed by PCR amplification. Amplified fragments were sequenced to verify their integrity and were then inserted into the vector pCR to obtain the recombinant plasmids pCR-50sp and pCR-57sp. These plasmids were used to transfect cultured cell lines of different origin to test their susceptibility to HIV-1 infection. Control cells were transfected with the pCR vector alone.

**Cell cultures.** Jurkat T cells were used as representative of cells naturally permissive to HIV infection. The B cell line BC-3, derived from PEL and chronically infected with HHV-8, was used as representative of HHV-8 target of infection and because of its permissiveness to HHV-8 (Merat et al., 2002). For non-permissive cells, a neuronal cell line derived from a human glioblastoma (A172) was used. H9 and C8166 T cell lines were used, respectively, for HIV-1 production and titration.

For infection of neuronal monolayers, A172 cells were trypsinized, centrifuged and suspended at a density of 5 × 10\(^4\) cells ml\(^{-1}\) in complete DMEM in the presence of HIV-1 at an m.o.i. of 1:10 or 1:100. Samples of 5 × 10\(^5\) cells were then seeded in sterile flasks and incubated at 37°C in 5% CO\(_2\) for 24 h. Infected cell monolayers were subsequently washed twice with PBS and re-fed with fresh medium. Cell and supernatant samples were collected at different times (0, 3, 7, 14, 21 and 28 days p.i.). Supernatants were centrifuged for 10 min at 1250 r.p.m. to exclude cell contamination and then treated as described above. Cell samples were obtained by trypsinization and centrifugation, followed by washing of the cell pellet with PBS prior to freezing as described above.

**PCR and RT-PCR analyses of infected cells.** DNA and RNA analyses of cell pellets were performed by PCR and RT-PCR,
respectively. Genomic DNA was extracted from $5 \times 10^6$ cells by phenol/chloroform extraction, as described previously (Di Luca et al., 1994). Total RNA was extracted with RNazol B (Tel Test), as recommended by the manufacturer. DNA contamination was eliminated by three cycles of digestion with 20 units RNase-free DNase (Roche) at room temperature for 30 min. The complete absence of DNA contaminants was checked by PCR amplification of 200 ng total RNA with human $\beta$-actin primers. Retrotranscription was carried out on 2 $\mu$g total purified RNA, using dNTP, random hexamer primers (Roche) and 20 units of AMV reverse transcriptase (Ambion). The presence of proviral HIV-1 DNA and virus transcripts was analysed by nested PCR of regions corresponding to the tat and gag genes on 100 ng DNA or 200 ng RNA. The primers for tat detection in first-round PCR were 5'-GAAGCATCGAGAAGTCAGGC-3' (nt 5864–5884) and (reverse) 5'-ACCTTCTTCTTATTCCTTCGGG-3' (nt 8436–8413), with the following amplification protocol: 94°C for 5 min, 35 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min, followed by a 10 min extension at 72°C, with an amplification product of 2572 bp for DNA and 240 bp for cDNA. For second-round PCR, a semi-nested reaction was performed using the same reverse primer and primer 5'-CTATGCGAGAAGAGCGG AGACA-3' (nt 5969–5992). The following conditions of amplification were used: 94°C for 5 min, 35 cycles of 94°C for 1 min, 63°C for 1 min and 72°C for 1 min, followed by a 10 min extension at 72°C, to obtain an amplified fragment of 2467 bp and 135 bp, respectively, for DNA and cDNA.

To detect gag, first-round PCR amplified a fragment of 438 bp, with primers 5'-GTACATCAGCAGCATCAC-3' (nt 1218–1237) and reverse 5'-GTCCCGTCTATTGCTCAGA-3' (nt 1656–1637), with the following thermal cycles: 94°C for 5 min, 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, followed by an extension at 72°C for 10 min. Nested primers were 5'-CATATTCAAGAAGGAGCCAC-3' (nt 1312–1331) and reverse 5'-GGTCCCGTCTATTGCTCAGA-3' (nt 1507–1488), and the amplification conditions were 5 min of denaturation at 94°C, 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s, with a single 10 min extension at 72°C, to amplify a 195 bp fragment. Amplification of the house-keeping $\beta$-actin gene was used as a control, as described previously (Caselli et al., 2001). The enzyme used for PCR was Taq polymerase (Perkin-Elmer). PCR products were run in a 1:5% agarose gel and visualized by ethidium bromide staining.

Quantification of HIV-1. Production of HIV in culture supernatants was monitored by measuring p24 antigen production and by titration of infectious particles. The presence of p24 was assessed by titration of cell supernatants on C8166 cells, as prescribed in the manufacturer’s instructions. Release of infectious virus was measured by titration of cell supernatants on C8166 cells, as described for virus stock production. Replication-competent virus associated with each cell preparation was evaluated by co-culturing 5 x 10^5 cells with 5 x 10^6 C8166 susceptible cells (indicator T cells). Lymphoid or adherent cell samples were collected by centrifugation or trypsinization to eliminate culture medium, which was tested separately. Productive infection was evaluated by observation of syncytium formation and p24 determination after 7 days of co-culture.

Analysis of HIV-1 receptors. Cell clones transformed with ORF50 or ORF57 were tested for the expression of CD4, CXCR-4 and CCR5 receptors both by Northern blot and by FACS analyses.

For Northern blot analysis, poly(A)+ RNAs were extracted from cell clones (mRNA isolation system, Roche) and hybridized with probes obtained by PCR amplification, specific for the CD4, CXCR-4 or CCR5 receptor. Blots were also hybridized with a $\beta$-actin probe to provide an mRNA loading control.

Cell surface expression of CD4, CXCR-4 and CCR5 receptors was analysed by flow cytometry. Specific fluorochrome-conjugated antibodies reacting with CD4, CXCR-4 and CCR5 (Becton-Dickinson) were used. Briefly, 10^6 cells were suspended in cold PBS and incubated for 30 min on ice with the appropriate anti-receptor antibody. Cells were washed twice with PBS and analysis was performed with a FACScan Flow Cytometer (Becton-Dickinson).

Statistical analysis. Statistical analysis of collected data were performed by Student’s $t$-test.

RESULTS

Infection of susceptible cells

To determine the effect of ORF50 on HIV infection of susceptible cells, Jurkat clones stably transformed by ORF50 or ORF57 were infected with HIV at different m.o.i. Briefly, Jurkat cells were transfected with constructs expressing ORF50 and ORF57 or with control plasmids, as described in Methods, and selected by G418 treatment. Drug-resistant clones were characterized by PCR and RT-PCR to assess the presence and transcription of ORF50 or ORF57 (data not shown). Clones transcribing recombinant genes at the highest levels were infected with HIV. The day before infection, G418 was removed from the culture and cells were cultivated in the absence of the selection drug for the complete duration of the experiment, in order to prevent interference with HIV infection. The results of p24 analysis, shown in Fig. 1(a, b), demonstrate that ORF50 has an enhancing effect on HIV infection. The increase was particularly evident at an m.o.i. of 1:100 000 (Fig. 1b), where the presence of ORF50 induced maximal p24 release as early as 12 days p.i., whereas control cells yielded measurable levels of p24 only at 20 days p.i. and did not reach the amount released by the cells transformed with ORF50. At higher m.o.i., 1:10 000, the effect of ORF50 was still evident but was limited to an earlier appearance of the plateau phase. ORF57 did not have any effect on p24 release. All differences observed in p24 release between ORF50 clones and control, or ORF57 clones, were statistically significant ($P<0.001$).

Titration experiments showed that the presence of ORF50 caused a substantial enhancement in the production of HIV infectious particles (Fig. 1c, d), confirming results obtained by p24 detection.

Analysis of HIV proviral DNA by PCR detection of tat and gag showed higher loads in ORF50 clones compared to control cells, particularly at early times p.i. (Fig. 2). Proviral DNA was detected as early as 4 days p.i. in Jurkat ORF50 clones at both m.o.i., whereas it was not visible in control groups until 8 days p.i. (at an m.o.i. 1:10 000, data not shown) and 12 days p.i. (at an m.o.i. 1:100 000); results were confirmed by RT-PCR analysis of transcription. Transcription of proviral DNA was detected as early as 4 days p.i. in Jurkat clones transformed with ORF50 and increased throughout the duration of the experiment. Parental cells showed the presence of transcripts only at 12 days p.i. The presence of ORF57 did not have any significant effect on HIV provirus load and transcription (data not shown).
Infection of permissive cells of the B lineage

Stable clones of BC-3 cells stably transformed with ORF50 or ORF57 were obtained and characterized by PCR and RT-PCR, as described for Jurkat cells, and were infected with HIV-1 at different m.o.i. (from 1:10 to 1:10,000). The release of p24 in BC-3 cells was evident even in control pCR-transfected cells, confirming that the BC-3 cell line is susceptible to HIV-1 infection (Merat et al., 2002). However, infected cells did not develop cytopathic effect. At high m.o.i. (from 1:10 to 1:100), all cell groups expressed and released similar amounts of p24 antigen (data not shown). However, at lower m.o.i. (1:1000 and 1:10,000), the presence of ORF50 resulted in a marked increase in p24 release (Fig. 3a). The increase was present also in ORF57-transformed cells. Analysis of provirus load and transcription showed higher levels of positivity in ORF50 clones compared to control cells (Fig. 2), confirming that even the parental BC-3 cell line is permissive to virus infection. Expression of ORF50, and ORF57 (data not shown), resulted in the earlier detection of HIV mRNA.

**Fig. 1.** (a, b) p24 production in stable clones of Jurkat cells expressing ORF50 or ORF57. Control cells were transformed with pCR. After selection, clones were infected with HIV-1 at an m.o.i. of 1:10,000 and 1:100,000. Culture supernatants were collected at the reported times and assessed for p24 release. Results are expressed as pg ml⁻¹ of p24 antigen, measured with a reference curve, and represent the mean of duplicate samples of three different experiments. (c, d) Quantification of HIV-1 in Jurkat clones infected with HIV-1 at an m.o.i. of 1:10,000 or 1:100,000. Culture supernatant was collected at the reported times and tested for infecting virus particle release by direct titration on C8166 target T cells. Results are expressed as CCID₅₀ ml⁻¹ and represent the mean of quadruplicate samples of two different experiments.

**Fig. 2.** Presence and transcription of HIV-1 proviral DNA. Analyses were performed by PCR and RT-PCR, respectively, on DNA and RNA extracted from infected cells at different days p.i. using primers specific HIV-1 gag. Similar results were obtained analysing the presence of tat DNA and RNA (data not shown). Parental cells, transformed with only the pCR vector (control) and clones stably transformed with pCR-50sp, were infected with HIV-1 at an m.o.i. of 1:100,000 (Jurkat cells), 1:1000 (BC-3 cells) and 1:10 (A172 cells). DNA extracted from Jurkat cells collected at complete cytopathic effect was used as a positive PCR control, whereas negative control consisted in DNA from uninfected cells.
HIV replication was confirmed by titration of the culture supernatants, measured by direct supernatant titration on C8166 target cells (Fig. 3b). Infectious virus was detected in the supernatant of ORF50-transformed BC-3 cells infected with HIV at all m.o.i. of 1:1000 (a, c) and 1:10 (b). (a) p24 analysis. Results are expressed as pg ml⁻¹ and represent the mean of duplicate samples from two different experiments. (b) HIV quantification by direct titration of culture supernatants on C8166 target cells. Results are expressed as CCID₅₀ ml⁻¹ and represent the mean of quadruplicate samples from two different experiments. (c) p24 production in co-infection experiments; 5 × 10⁵ infected B cells were co-cultured with 5 × 10⁵ C8166 target cells for 7 days, p24 production in culture supernatants was then measured. Results are expressed as pg ml⁻¹ and represent the mean of duplicate samples from two different experiments.

Fig. 3. HIV infection in BC-3 B cells. BC-3 clones and parental cells were infected with HIV at an m.o.i. of 1:1000 (a, c) and 1:10 (b). (a) p24 analysis. Results are expressed as pg ml⁻¹ and represent the mean of duplicate samples from two different experiments. (b) HIV quantification by direct titration of culture supernatants on C8166 target cells. Results are expressed as CCID₅₀ ml⁻¹ and represent the mean of quadruplicate samples from two different experiments. (c) p24 production in co-infection experiments; 5 × 10⁵ infected B cells were co-cultured with 5 × 10⁵ C8166 target cells for 7 days, p24 production in culture supernatants was then measured. Results are expressed as pg ml⁻¹ and represent the mean of duplicate samples from two different experiments.

Co-cultivation of HIV-infected BC-3 clones with C8166 cells resulted in a massive production of HIV, shown by the appearance of large syncitia as well as by the production of p24 (Fig. 3c).

Infection of non-permissive neuronal cells

To determine the effect of ORF50 on HIV infection of non-permissive neuronal cells, A172 cells, derived from human glioblastoma, were infected with HIV-1 at an m.o.i. of 1:10 and 1:100, after transfection with HHV-8 ORF50 plasmid and selection of stable clones. Parental cells and ORF57-transformed clones did not produce p24 upon HIV-1 infection (Fig. 4a). Instead, expression of ORF50 resulted in the production of p24 throughout the 28 days of observation, even if the protein was produced in low amounts (peak 49 pg ml⁻¹) when compared to T cells or PEL-derived infected cells (Fig. 4a). HIV proviral DNA was detected by PCR in ORF50-transformed clones, 3 and 7 days p.i., but not in parental or ORF57-transformed cells. Analysis by nested PCR showed that cells expressing ORF50 were still positive 14 days p.i. and that controls were faintly positive only at day 3 p.i. (Fig. 2). RT-PCR analysis of gag and tat transcripts confirmed that HIV proviral DNA was transcriptionally active in ORF50-transformed clones, whereas controls contained faint traces of transcripts, revealed only 3 days p.i. by nested RT-PCR.

Titration assays of culture supernatants showed that HIV-infected A172 cells did not release detectable amounts of infectious virus (Fig. 4b). Nevertheless, co-infection experiments showed that ORF50-expressing cells, but not parental cells, were able to transmit HIV-1 to susceptible C8166 cells by direct contact, as demonstrated by the production of p24. This effect was present at 21 days p.i. and disappeared at day 28 p.i. (Fig. 4c). Therefore, the presence of ORF50 allowed HIV to persist longer than in parental cells (Fig. 2) and resulted in low levels of transient replication (Fig. 4c).

Analysis of cell surface receptors for HIV

T, B and glial cells transfected with ORF50 showed increased susceptibility to HIV infection. To determine whether ORF50 upregulated HIV receptors and co-receptors, transcription of CD4, CXCR-4 and CCR5 was analysed by Northern blot using molecular probes specific for each transcript. CD4 and CXCR-4 were constitutively transcribed in Jurkat and BC-3 cells. The expression of ORF50 slightly increased CD4 and CXCR-4 transcription but had no effect on CCR5 (Fig. 5). The glioblastoma cell line A172 was negative for CD4 and CXCR-4, even in the presence of ORF50, but CCR5 transcription was upregulated in ORF50-transformed clones (Fig. 5). Results were confirmed by FACS analysis using specific anti-receptor antibodies (data not shown). Transfection of ORF50 and ORF57 resulted in the increase in CD4⁺ BC-3 cells. Likewise, approximately 30% of BC-3 cells expressed CXCR-4 and in the presence of ORF50 or ORF57, the number of positive cells showed a moderate increase, respectively, to 45 and 41% (data not shown). CCR5 was expressed only in a minority of cells, even in the
presence of ORF50. Jurkat cells had high basal expression of both CD4 and CXCR-4, and the presence of ORF50 resulted in only a slight increase in expression. A172 cells did not express CD4 or CXCR-4 on their surface and HHV-8 ORFs had no effect.

**DISCUSSION**

ORF50 is an immediate-early gene of HHV-8 with important transactivating capabilities; its expression is necessary for virus reactivation and lytic replication (Gradoville et al., 2000; Lukac et al., 1998, 1999; Sakakibara et al., 2001; Song et al., 2001). Recently, we reported that ORF50 synergizes with HIV-1 tat at a post-transcriptional level (Caselli et al., 2001), enhancing transactivation of LTR. Results suggested that expression of ORF50 may render active small amounts of Tat, which would not necessarily have significant effects per se. The relevance of this finding lies within the context of reciprocal interactions between HHV-8 and HIV, since it is known that both viruses can infect the same cell types, including B cells and monocytes (Moir et al., 1999, 2000; Monini et al., 1999), HIV replication can stimulate HHV-8 production (Moore et al., 2000; Varthakavi et al., 1999) and it can support further the ‘in vitro’ productive infection of PEL cell lines chronically infected with HHV-8 (Harrington et al., 1997; Varthakavi et al., 1999). Therefore, even small amounts of both viruses, or few co-infected cells, could result in significant biological interactions.

To verify whether the molecular interactions observed previously have important effects on HIV replication and biology, cell lines of different origin were transfected with ORF50, clones expressing stable levels of specific mRNA were selected and infected subsequently with HIV. These findings show that ORF50 increases cell susceptibility to HIV infection in cells of different origin. Jurkat T cells exhibited a quicker course of HIV infection upon ORF50 transfection (Fig. 1a), higher levels of HIV Gag polypeptide p24 (Fig. 1b) and enhancement in the production of infectious virus (Fig. 1c, d). Stable transformation of BC-3 cells with ORF50 resulted in high levels of p24 and substantial release of infectious HIV in the culture supernatant (Fig. 3). Finally, ORF50 induced susceptibility to HIV and allowed restricted virus replication in A172 glial cells (Fig. 4).
In particular, experiments in Jurkat cells showed that ORF50 stimulates HIV replication in CD4+ T cells, the primary target of retrovirus infection. Stimulation of HIV replication was not associated with a generic state of cell activation, as shown in control experiments performed in the presence of TPA (data not shown). Furthermore, ORF57, another powerful HHV-8 transactivator, had no effect upon p24 production. As shown in Fig. 1(c, d), the presence of ORF50 results in several fold increments of virus titre, supporting the hypothesis that HHV-8 might be particularly important in triggering HIV replication and amplification. In fact, the effects of ORF50 are particularly evident when HIV ‘in vitro’ infectious doses are low (m.o.i. of 1 : 10-000 and 1 : 100 000).

The effect of ORF50 on HIV is particularly interesting in B cells, which can be infected by both viruses. It was reported recently that BC-3 cells, derived from a human primary effusion lymphoma and chronically infected with HHV-8, are permissive to HIV infection (Merat et al., 2002). Our results confirm that BC-3 cells support HIV replication, as shown by p24 production and release of infectious virus in culture supernatant. In fact, the stable expression of ORF50 resulted in the enhancement of HIV replication in BC-3 cells (Fig. 3), with a remarkable increase over control cells. However, the presence of ORF57 caused an increase in HIV replication. A plausible explanation is that ORF57 does not have a direct effect on HIV, as shown by the absence of enhancement in Jurkat cells stably transformed with ORF57. Instead, it is likely that the effect observed in BC-3 is associated with the activation of HHV-8 replication induced by ORF57 (Bello et al., 1999; Gupta et al., 2000; Kirshner et al., 2000) and the consequent expression of ORF50 and other virus functions. These results confirm that cells of the B lineage might act as a reservoir for HIV (Moir et al., 1999) and suggest that HHV-8 infection might increase HIV replication. In fact, B cells can potentially interact and pass the virus on to T cells, both in the peripheral blood and after migration to tissues.

Experiments in A172 undifferentiated glioblastoma cells show that this cell line maintains the characteristic non-permissiveness to HIV infection, even in the presence of ORF50. Analysis of p24 showed only a transient, low level production of protein (Fig. 4a) and no infectious virus was released in the culture supernatant (Fig. 4b). It was reported previously that the block to HIV infection in A172 cells possibly results from a restriction of HIV entry (Jault et al., 1994). Our observation that parental A172 cells infected with HIV are negative for proviral DNA and do not transcribe tat and gag (Fig. 2) supports this hypothesis. However, HIV enters into ORF50-transformed A172, contrarily to what is observed in parental cells, as shown by the detection of viral DNA and transcripts (Fig. 2). Furthermore, co-cultivation of infected A172 with uninfected C8166 resulted in detectable production of p24 only in the presence of ORF50 (Fig. 4c). This observation could be relevant for the transmission of HIV infection to adjacent cells. In fact, both HIV and HHV-8 are detected frequently in brain tissue. Chan et al. (2000) reported that over 60% of healthy brains contain HHV-8 sequences and brain tissue of HIV-positive patients is commonly infected with HIV (Levy et al., 1985; Nuovo et al., 1994). Most likely, under normal conditions, HHV-8 is latent in brain tissue but the detection of viral sequences in the cerebrospinal fluid of AIDS patients (Broccolo et al., 2000) suggests that the virus can occasionally replicate in the CNS. Therefore, it is possible that foci of HHV-8 lytic infection within the CNS may induce HIV replication, sufficient to promote HIV diffusion in the cerebral district.

Further experiments currently ongoing in our laboratory show that the enhancing effect of ORF50 on HIV replication is not restricted to specific cell types but takes place also in monocytic cell lines (E. Caselli and D. Di Luca, unpublished results). The relevance of the observed in vitro interactions to the in vivo clinical situation, namely whether active HHV-8 infection results in increased loads of HIV, still remains to be assessed. Only few studies have compared HIV and HHV-8 virus loads in infected patients but there are some indications that HHV-8 load is associated with higher levels of HIV. Brodt et al. (1998) found that KS patients show an accelerated clinical course of HIV infection. Tedeschi et al. (2001) reported that there is an association between HHV-8 DNA and HIV RNA virus loads and suggested that the two viruses might have synergistic interactions. Similarly, Min & Katzenstein (1999) described that levels of HIV RNA were associated strongly with HHV-8 DNA detection and that levels of HIV RNA were higher in subjects with increased copy numbers of HHV-8, further suggesting synergistic interactions between the two viruses. Finally, Mercader et al. (2001) showed that HIV replication is increased significantly in both in vitro and in vivo models of co-infection. More specifically, HIV-infected cells were injected in the presence or absence of HHV-8 in normal skin transplanted onto SCID mice and RT assays and p24 ELISA tests showed significantly increased values of HIV replication in animals injected with both viruses.

In conclusion, ORF50 has an enhancing effect on HIV replication in all cell lines analysed. ORF50 increases HIV replication in permissive cells (Jurkat and BC-3) and induces susceptibility and transient permissiveness in non-susceptible A172 cells. The mechanisms responsible for ORF50-mediated induction of HIV replication remain to be identified. In fact, the moderate increase in CD4 and CXCR-4, detected in ORF50-transformed Jurkat and BC-3 cells, is not sufficient to account for the elevated sensitivity to HIV infection and does not explain the transient permissivity observed in A172 cells. Likewise, ORF50 does not seem to regulate HIV transcription directly. In fact, we reported previously (Caselli et al., 2001) that ORF50 interacts synergistically with HIV-1 Tat in LTR transactivation at a post-transcriptional level. Therefore, the effect of ORF50 on HIV replication seems to be modulated by the intracellular environment. We plan to use DNA microarray technology.
to determine which alterations of cell gene transcription are induced by HHV-8 ORF50.

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REFERENCES


Grandadam, M., Dupin, N., Calvez, V. & 8 other authors (1997). Exacerbations of clinical symptoms in human immunodeficiency virus type 1-infected patients with multicentric Castleman’s disease are associated with a high increase in Kaposi’s sarcoma herpesvirus DNA load in peripheral blood mononuclear cells. J Infect Dis 175, 1198–1201.


