A role for human immunodeficiency virus type 1 Vpr during infection of peripheral blood mononuclear cells

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Studies analysing human immunodeficiency virus type 1 replication in primary cells have demonstrated that Vpr, although dispensable, plays a role along with the matrix (MA) protein in allowing nuclear localization of viral preintegration complexes in non-dividing monocyte-derived macrophages (MDMs). In the current study, experimental infection conditions to analyse the role of Vpr, independently of MA, during infection of PHA/IL-2-stimulated peripheral blood mononuclear cells (PBMC) were designed. It was shown that the absence of Vpr results in a subtle effect on virus production in long-term infection. PCR analysis of the steps of virus retrotranscription during a single cycle of replication in stimulated PBMC revealed that the absence of Vpr alone correlates with an impairment in the nuclear localization of viral DNA. Our data indicate that Vpr is involved in the virus life-cycle during infection of dividing PBMC, presumably as it is during infection of MDMs.

Human and simian immunodeficiency viruses (HIV and SIV) encode, in addition to structural proteins Gag, Pol and Env, some regulatory proteins which appear to be dispensable for replication in certain cell types. These include Vpr, which is a virion-associated protein (Kondo & Göttlinger, 1995; Lavallée et al., 1994, Lu et al., 1995; Paxton et al., 1993), strongly suggesting that it could play a role early upon infection. Although the overall benefit for virus replication is not fully understood, Vpr has been reported to act as a transactivator protein of several heterologous promoters including the HIV-1 long terminal repeat (Agostini et al., 1996; Cohen et al., 1990; Wang et al., 1995), to induce the terminal differentiation of some cell types and cause accumulation in the G2 + M stage of the cell cycle (for a review see Emerman, 1996). During the virus life-cycle in non-dividing cells, Vpr with respect to the HIV-1 nuclear localization signal (NLS) of the matrix (MA) protein, has been demonstrated to play a crucial role during the virus replication in monocyte-derived macrophages (MDMs) in allowing the nuclear localization of viral preintegration complexes (Bukrinsky et al., 1993; Heinzinger et al., 1994). Recent work, however, has reported that mutations within both MA and Vpr do not abolish infectivity in MDMs (Freed et al., 1995). Experiments carried out to study the role of Vpr during virus replication in proliferative cells also have revealed conflicting results. A critical role for Vpr has been reported in some cases of peripheral blood lymphocyte infection (Balliet et al., 1994), while others have reported that its role is negligible (Connor et al., 1995; Dedera et al., 1989; Ogawa et al., 1989). In the present study, we further examined the role of Vpr, independently of the role of MA, during infection of stimulated peripheral blood mononuclear cells (PBMC). Our data suggest that Vpr, although dispensable for virus replication in stimulated PBMC, likely plays a role in the nuclear localization of viral DNA.

To assess the importance of Vpr on HIV-1 replication in PHA/IL-2-stimulated PBMC, we analysed the relative infectivity of AD8 Vpr+ and Vpr− viruses. To obtain the Vpr− molecular clone from pAD8 (Theodore et al., 1996), an overlapping double-stranded oligonucleotide containing NcoI and EcoRI restriction sites plus stop codons in the three open reading frames (upper strand, 5′ CATGTAATTAATTAACCTGGAAAAG 3′; lower strand, 5′ AATCTTTCGAGTTAATTAACCTGGAAAAG 3′) was inserted into NcoI and EcoRI restriction sites located into the vpr gene to replace the corresponding restriction fragment of pAD8. The resulting Vpr protein encodes only the first 37 amino acids of Vpr. Virus stocks were obtained from cell-free supernatant of 293T cells transfected with viral DNAs. PBMC were cultivated in RPMI supplemented with 15% foetal calf serum and 200 U/ml IL-2 (CHIRON) and infected on day 3 or 6 after PHA stimulation as previously described (Balliet et al., 1994). Infection of cells was performed with equal amounts of Vpr+ and Vpr− viruses.
Fig. 1. Replication kinetics of Vpr+ and Vpr− AD8 in PHA/IL-2-stimulated PBMC. Virus stocks prepared by transfecting 293T cells with pAD8 wild-type or mutated for Vpr were calibrated to infect 1 x 10⁶ cells starting from 40,000 c.p.m. equivalent RT activity for the undiluted sample. Serial tenfold dilutions were used to infect the same number of cells in parallel. Virus replication was followed by measuring RT activity in cell-free supernatant twice weekly for 21 days. Data are representative of two independent experiments obtained from infections of PBMC from two different donors.

Fig. 2. PCR analysis of reverse transcripts produced in PBMC after infection with AD8 Vpr+ and Vpr−. 1 x 10⁶ cells were infected with 100 TCID₅₀ of each virus as titrated on PBMC (corresponding to 1 ml of the 10⁻¹ dilution). Undiluted or diluted tenfold samples of cellular DNA from infected cells were subjected to one round of PCR with primers specific for early reverse transcripts (R/U5) and late reverse transcripts (R/gag). Following amplification, products were analysed by Southern blotting and hybridized with a specific 32P-labelled oligonucleotide probe. As a control, infection was performed at 4 °C. Where indicated, AZT was added to the culture before, during and after infection. β-actin controls allowed the demonstration of relative amounts of cellular DNA in each sample. Linearity of the PCR amplification was determined using serially diluted DNA from 8E5 cells (Folks et al., 1986) that contain a single integrated provirus per cell.

monitored as serial tenfold dilutions of equivalent virus stocks estimated by measuring the reverse transcriptase (RT) activity. Virus production was monitored twice a week by measuring RT activity in infected cell culture supernatants (Fig. 1). Replication of Vpr+ and Vpr− viruses in PHA/IL-2-stimulated PBMC showed that, in the absence of Vpr, virus production was reduced and delayed, although the difference between Vpr+ and Vpr− growth kinetics was not particularly large. There was no difference in virus production for Vpr+ and Vpr− viruses with the higher TCID₅₀, while a decrease and a delay in the peak of RT production appeared with the lower TCID₅₀. These data indicate that, in dividing cells, the effect of Vpr on virus replication, although modest, was detectable only when low doses of virus were used.

To confirm that Vpr indeed plays a role in virus replication, as observed during long-term infection, we analysed viral DNA synthesis in stimulated PBMC shortly after infection (Fig. 2). Virus-containing supernatants were treated with DNase I (20 µg/ml) for 30 min at 30 °C in the presence of 10 mM MgCl₂. PBMC were infected with Vpr+ and Vpr− virus stocks (100 TCID₅₀ for 10⁶ cells). After infection, cells were treated with Trypsine XIII (15 µg/ml) for 15 min at 37 °C, and further cultured with addition of AZT (5 µg/ml) where indicated. The replication efficiency was quantified shortly after infection by measuring amounts of newly synthesized viral DNA in PBMC target cells at 3, 8, 24 and 72 h post-infection. At designated times, total cellular DNA was extracted and used as a PCR template to amplify products of
reverse transcription as previously described (Courcoul et al., 1995). Synthesis of the minus-strand cDNA was detected with primer pairs R/U5 and R/gag and corresponds to early and late products of the reverse transcription, respectively. At 3 h post-infection, roughly similar levels of early retrotranscribed viral DNA products were detected for Vpr+ and Vpr− viruses, indicating an equivalent entry of viruses in infected cells. As controls, infections were performed either at 4 °C or in the presence of AZT added before and during infection to ensure that cDNA products detected in acutely infected cells were the result of both virus infection and de novo DNA synthesis rather than of carry-over proviral DNA in the inoculum. Amounts of late products of reverse transcription showed no significant difference between Vpr+ and Vpr− virus-infected target cells at 8 h post-infection, suggesting that the reverse transcription process is not significantly affected in the absence of Vpr. At 24 h and 72 h post-infection, DNA synthesis increased, likely due to new rounds of infection, but remained significantly impaired in Vpr− virus-infected cells. These data indicate that the expression of Vpr results in the accumulation of larger amounts of viral DNA and plays a positive role on the propagation of infection in stimulated PBMC. To analyse the proliferative status of stimulated PBMC used in this study, we measured the rate of incorporation of [3H]thymidine during a set period of time. As expected, we observed that unstimulated PBMC failed to incorporate any DNA precursor, while stimulated PBMC did, although with a rate half that of immortalized T cell lines such as SupT1 (data not shown). These data indicate that stimulated PBMC are indeed dividing cells. In addition, it has been reported that reverse transcription in mitogenic-stimulated T cells is completed within less than 6 h post-infection, while no extension of the reverse transcription process is observed in quiescent cells (Zack et al., 1990, 1992). In agreement with these data, we observed that complete reverse transcripts identified with R/gag primers are detected within 3 h following infection, suggesting that cells are not in a quiescent stage. We also determined the cell cycle distribution by measurement of the DNA content of the cells at the time of infection, and found that approximately 30% of the cells were in the G2 + M phase of the cell cycle (data not shown).

We next performed experiments in which virus replication was analysed during a single cycle of infection. PHA/IL-2-stimulated PBMC were infected with equivalent Vpr+ and Vpr− virus stock, treated 8 h post-infection with AZT and incubated for 36 h to allow completion of the virus life-cycle. We quantified virus production in PBMC cell-free supernatant by RT–PCR amplification of viral genomic RNA. Vpr+ and Vpr− viruses were concentrated by ultracentrifugation, lysed in 200 μl RNAaseOL (Bioprobe), and RNAs were purified as instructed by the manufacturer and retrotranscribed using avian myeloblastosis virus RT (Promega) in the presence of antisense primer gag. Following retrotranscription, DNA products were diluted in fivefold serial dilution and then amplified using R/gag primers. As shown in Fig. 3(a), the comparison of the amounts of viral RNA in Vpr+ and Vpr− virus-infected supernatant from PBMC demonstrates a clear decrease in the RT–PCR signals in Vpr− viruses, indicating that the production of virus is impaired in the absence of Vpr. This difference in virus production could explain the difference in the propagation of infection observed in the long-term infection of PBMC. To further examine which step of the virus life-cycle was impaired, we analysed steps of the reverse transcription process in infected cells (Fig. 3b). Amounts of early and late reverse-transcribed products remained quite similar for Vpr+ and Vpr− viruses and provided useful internal controls for evaluating relative amounts of 2 LTR circle products. The 2 LTR circle products are formed only after completion of viral DNA synthesis and localization to the nucleus (Pauza et al., 1994). Analysis of relative amounts of 2 LTR circle products clearly showed an impairment in the nuclear localization of viral DNA in cultures infected with Vpr− viruses. Failure to detect 2 LTR circle products in cells infected with Vpr− viruses is likely to be due to undetectable amounts rather than a total blockage in the nuclear transport of viral cDNA, since cells were able to produce virus particles. Similar results were obtained in three independent experiments.
These data indicate that, during infection of PBMC, the completion of reverse transcription, including nuclear localization of complete viral cDNA, is impaired in the absence of Vpr.

In this paper, we demonstrated that Vpr plays a positive role in virus replication in the context of a spreading infection in stimulated PBMC. Previous studies analysing the virus life-cycle during infection of non-dividing cells have demonstrated that viruses simultaneously defective in both Vpr and the NLS of MA protein were impaired in the nuclear localization of viral preintegration complexes in the absence of mitosis (Bukrinsky et al., 1993; Heinzinger et al., 1994). The role of Vpr alone in this process has not been demonstrated, although the authors suspected the two signals for nuclear localization to be redundant and additive (Heinzinger et al., 1994). Here, we observed that replication in stimulated PBMC of Vpr-defective viruses resulted in a delay in virus production, which could be observed only when infections were performed at a low m.o.i. This delay could be compensated for by infecting cells with increasing amounts of mutant particles. The effect of the m.o.i. on the phenotype could explain why previous studies (Balliet et al., 1994; Bukrinsky et al., 1993; Connor et al., 1995; Dedera et al., 1989; Ogawa et al., 1989) have failed to observe any influence of Vpr on virus replication in PBMC. We thus propose that Vpr, although dispensable for virus replication in PBMC, could nevertheless play a role in optimizing one or more of the steps in the virus life-cycle. Indeed, our PCR analysis performed after a single cell cycle of infection with Vpr− viruses revealed a defect in the nuclear localization of viral DNA, indicating that this step of the virus life-cycle required, at least in part, the presence of Vpr. Recently, similar data indicating that SIVagm Vpr is required for nuclear import of viral DNA in macaque stimulated PBMC have been reported (Campbell & Hirsch, 1997). The mechanism by which Vpr participates in the nuclear localization process of viral DNA, however, remains to be elucidated. One possibility could be that Vpr plays a role in the stability of the core preintegration complex by preserving its integrity long enough to make it into the nucleus. Alternatively, Vpr could be required in the molecular process inducing the nuclear import per se. Since stimulated PBMC infected with HIV-1 Vpr-defective viruses were able in the end to propagate infection, it appeared that the phenotype observed consisted only of a transient delay in virus replication. Our experimental conditions (i.e. low m.o.i. and short time for infection) allowed us to observe the slight contribution of Vpr in the nuclear localization process. It is likely that the slow rate of cell division of stimulated PBMC contributed towards the amplification of the phenotype observed. Supporting this hypothesis, we failed to demonstrate a role for Vpr in the HIV-1 DNA nuclear import during infection under similar conditions in highly dividing T cells, such as SupT1 (data not shown). It is likely that our experimental infection conditions unveiling the role of Vpr during infection of dividing PBMC are close to the in vivo features of virus spread, suggesting that the HIV-1 Vpr protein might be required during infection of the two target cell types, MDMs and PBMC.

We want to thank Eric Freed and Gilles Querat for critical reading of the manuscript and helpful comments. This work was supported by INSERM and grants from the French Agency against AIDS (ANRS).

References


Received 19 December 1997; Accepted 9 January 1998