An additional mechanism of growth restriction in T cell line H9 of human immunodeficiency virus type 1 isolates from asymptomatic homosexual men

Raghavan Balachandran, Mandaleshwar K. Singh and Phalguni Gupta*

Department of Infectious Diseases and Microbiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, PA 15261, USA

The replicative properties of human immunodeficiency virus type 1 (HIV-1) isolates from asymptomatic carriers (asymptomatic isolates) and AIDS patients (AIDS isolates) were examined in the human T lymphocyte cell line H9. In agreement with earlier reports the replication of asymptomatic isolates was restricted whereas AIDS isolates replicate well in H9 cells. PCR analysis of H9 cells infected with asymptomatic isolates showed transient gag DNA synthesis for up to 48 h post-infection. This transient DNA synthesis was much lower than the amount of DNA synthesized by the AIDS isolates. The reduction in DNA synthesis reflects a restriction during virus entry. We further analysed transient DNA synthesis by the asymptomatic isolates to investigate possible post-entry restriction mechanisms. The transiently synthesized DNA was present only in the unintegrated form and was not transported in to the nucleus, suggesting an additional restriction mechanism for asymptomatic isolates in H9 cell lines at a step post-DNA synthesis and prior to or during nuclear translocation of newly synthesized viral DNA.

Introduction

Infection with human immunodeficiency virus type 1 (HIV-1) is associated with diverse clinical outcomes ranging from the asymptomatic carrier state to full-blown AIDS. Analysis of biological characteristics of HIV-1 isolates from asymptomatic subjects and from AIDS patients (hereafter referred to as asymptomatic isolates and AIDS isolates, respectively) indicates distinct differences. We and others have shown that AIDS isolates are more virulent than most asymptomatic isolates with respect to their in vitro replication rates, ability to infect a wide variety of CD4+ T cell lines and induction of syncytia (Balachandran et al., 1991; Cheng-Mayer et al., 1988; Fenyo et al., 1988; Tersmette et al., 1989). Furthermore, transition from non-syncytium-inducing to syncytium-inducing HIV-1 isolates in infected patients has been shown to be associated with the development of AIDS (Koot et al., 1992). Several investigators have demonstrated that the envelope region of the HIV-1 genome carries genetic determinants responsible for tropism in T cell lines and syncytium induction (Cheng-Mayer et al., 1990; Cann et al., 1992; Hwang et al., 1991). The determinants for T cell tropism were further localized in the V3 region of outer envelope protein gp120. Cann et al. (1992) reported that the restriction of replication of asymptomatic isolates in H9 cells is determined by events prior to provirus formation. We have examined the replication of HIV-1 isolates from asymptomatic and AIDS patients in H9 cells. Unlike AIDS isolates, the asymptomatic isolates do not show productive infection in H9 cells and do not form syncytia in MT-2 cells. The asymptomatic isolates also replicate more slowly in phytohaemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) and produce lower levels of viral DNA, RNA and proteins when compared to AIDS isolates (Balachandran et al., 1991). The results presented here support a mechanism of growth restriction of asymptomatic isolates in T cell lines at a step prior to provirus formation as previously described. Our data show that asymptomatic virus infection produces transient viral DNA components which are deficient in translocation to the nucleus and integration into the host genome. Such a post-entry level defect may be responsible for the non-productive replication of asymptomatic isolates in H9 cells.

* Author for correspondence. Fax +1 412 624 4953.
e-mail pgupta1@vms.cis.pitt.edu

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Methods

Virus isolates. HIV-1 isolates were prepared from PBMC from five asymptomatic seropositive subjects and five AIDS patients enrolled in the Multicenter AIDS Cohort Study (MACS) and AIDS Clinical Trials Unit (ACTU) at Pittsburgh. Asymptomatic subjects used in this study were classified as CDC I and II, while all AIDS patients used were CDC group IV with AIDS defining clinical symptoms.

Virus isolates were propagated by infecting 1 x 10⁸ PHA-stimulated PBMC with 1 x 10⁵ c.p.m. reverse transcriptase units (RTU)/ml as described (Balachandran et al., 1988, 1991). Infection of H9 cells was carried out as described (Balachandran et al., 1988, 1991) except that the virus inoculum was incubated prior to infection with DNase I (80 U/ml) for 1 h at 37 °C in the presence of 10 mM-MgCl₂ and pelleted at 14000 g for 2 h at 4 °C in a microfuge (Levy & Shimabukuro, 1985).

Virus pellets were resuspended in 1 ml of RPMI 1640 with 20% serum and used as inoculum. The replication of HIV-1 in H9 cells was monitored by measuring p24 in culture supernatant using an antigen capture test (Dupont). The infectivity of an isolate was determined by infecting H9 cells with serial dilutions of HIV-1 that had been pre-incubated with DNase I as described above. After infection, cells were washed and incubated in medium for 20 h; 1 x 10⁵ cells were then harvested and subjected to amplification by PCR. The highest dilution which produced an HIV-1 gag DNA signal was considered to be the titre of the isolate (henceforth referred to as PCR infectious dose, PCID).

PCR. DNA PCR was performed on the lysed cell pellets using oligonucleotides SK38/39 (Wolinsky et al., 1989) as primers to amplify a 114 bp region of gag and primers M667/M844 (Zack et al., 1990) to amplify a 285 bp region spanning the 2LTR junction in circular HIV-1 DNA as previously described (Wolinsky et al., 1989; Gupta et al., 1992). PCR was performed for 30 repeated cycles of target DNA thermal denaturation (94 °C) for 30 s, oligonucleotide primer annealing (55 °C) for 30 s and Taq DNA polymerase-catalysed template extension (72 °C) for 45 s. One-third of the volume of the amplified reaction product was hybridized in solution to a specific ³²P end-labelled oligonucleotide probe (200 000 c.p.m./sample) derived from an internally conserved sequence in the amplified region (SK19 for the SK38/39 primer pair; KG1 (Gupta et al., 1992) for the M667/M667 and M844/M667 primer pairs). The oligonucleotide probe-target DNA was resolved by electrophoresis on a 10% polyacrylamide gel. The characteristic fragment of defined size was visualized by autoradiography after exposure to Kodak XAR film with intensifying screens at ~70 °C. DNA isolated from agarose gel was analysed by PCR for a conserved region of the HLA DQα locus using the primer pair GH26/GH27 (Wolinsky et al., 1989).

Unintegrated and integrated DNA were fractionated by a modification of the procedure described by Pang et al. (1990). Briefly, total (integrated and unintegrated) DNA (~3 μg) from the cell lysates was resolved by electrophoresis in 1% low melting agarose at 35 V for 48 h. Ethidium bromide stained DNA fragments larger than 15 kb (integrated) and DNA fragments smaller than 12 kb but larger than 4 kb (unintegrated) were excised from the gel. Gel slices were digested with Gelase (Epicenter Technologies) at 45 °C for 1 h and used directly for PCR amplification. DNA from 8E5 cells, a chronically infected T cell line containing one integrated copy of HIV-1 DNA per cell (Gendelman et al., 1987), served as a marker for integrated DNA.

Results

Five asymptomatic isolates and five AIDS isolates were examined for replication in H9 cells. An inoculum of 1 x 10⁵ c.p.m. RTU per 5 x 10⁶ H9 cells was used for all HIV-1 isolates. Fig. 1 shows representative results of H9 infection with one asymptomatic and one AIDS isolate. As reported earlier, all five AIDS isolates showed productive infection, producing > 1000 pg/ml of p24 within 6–8 days of infection; cells became chronically infected and produced virus (>10000 pg/ml) continuously for at least 6 weeks (Balachandran et al., 1991). In contrast, none of the five asymptomatic isolates produced significant amounts of virus (<100 pg/ml of p24) in H9 cells 12 days post-infection and also 21 days post-infection. Although these asymptomatic isolates did not productively infect H9 cells, they were infectious in PHA-stimulated donor PBMC (>2400 pg/ml). However, the amount of virus produced by the asymptomatic isolates in PBMC was consistently 5-10-fold lower than that produced by AIDS isolates (Sovo et al., 1995).

To investigate the mechanism of nonproductive infection by asymptomatic isolates in H9 cells, we analysed the synthesis of HIV-1 DNA by PCR using the gag specific primer pair SK38/39. Fig. 2 shows the results of PCR analysis of viral gag DNA synthesis in H9 cells infected with five asymptomatic and five AIDS isolates each with an inoculum of 1 x 10⁵ c.p.m./ml RTU. HIV-1 gag DNA was detected for all asymptomatic and AIDS isolates at 24 h post-infection. However, the asymptomatic isolates synthesized a considerably lower level of gag DNA as compared to AIDS isolates. From the copy number control included in the PCR assay, it is estimated that 10–25 copies of gag DNA were synthesized per 100000 cells infected with asymptomatic isolates compared to > 500 copies of HIV-1 gag DNA synthesized in the same
HIV growth restriction in a T cell line

Asymptomatic isolates

AIDS isolates

Fig. 2. Synthesis of HIV-1 DNA following infection of H9 cells with five asymptomatic isolates (1–5) and five AIDS isolates (1–5). A virus inoculum of 1 × 10^4 c.p.m. RTU/ml was used to infect H9 cells. At the indicated days post-infection 1 × 10^6 infected cells were harvested and analysed for HIV-1 DNA by PCR using primer pairs SK38/39. An amplified product of 114 bp is indicated.

Fig. 3. Synthesis of HIV-1 DNA in H9 cells infected with two asymptomatic isolates (1 and 2) and two AIDS isolates (3 and 4) with equal PCR infectivity titre. At the indicated days post-infection, 1 × 10^6 cells were harvested and analysed for HIV DNA by PCR using primer pair SK38/39.

number of cells by AIDS isolates. Furthermore, the low level of viral gag DNA synthesized during infection by the asymptomatic isolates decreased after 4 days and was not detectable 8–10 days post-infection. In contrast, high levels of viral gag DNA were detected in cells infected with all AIDS isolates throughout the infection period, even 16 days post-infection. The absence of viral gag DNA 1 h post-infection (day 0, Fig. 2) suggests that the viral DNA detected 20 h post-infection represents de novo viral DNA synthesis, rather than contaminating DNA in the viral inoculum. This suggestion was further substantiated by the absence of viral DNA synthesis (< 1 copy of gag DNA) in H9 cells infected with heat-killed virus (data not shown). The results in Fig. 2 show that a significant amount of viral DNA is still synthesized by asymptomatic isolates, indicating that an additional defect in replication post-DNA synthesis may be responsible for the lack of virus production.

In order to investigate the possibility that the difference in gag DNA synthesis between AIDS isolates and asymptomatic isolates could be due to a difference in infectivity of the inoculum between the two types of isolate, we examined viral DNA synthesis in H9 cells infected with two asymptomatic isolates (1 and 2) and two AIDS isolates (3 and 4) with equal PCR infectivity titres. Results shown in Fig. 3 indicate that although approximately equal levels of HIV-1 gag DNA were produced by two asymptomatic isolates as compared to two AIDS isolates within 24 h of infection, the gag DNA PCR signal intensity decreased at 48 h post-infection and disappeared by 8 days post-infection. In contrast, the levels of gag DNA produced by the two AIDS isolates increased progressively with time. This is further evidence for a defect in virus replication post-DNA synthesis.

In order to further analyse the restrictive infection process, we compared the state of integration of HIV-1 DNA in H9 cells infected with two asymptomatic isolates (1 and 2) and two AIDS isolates (3 and 4) at equal input m.o.i. values determined as PCID. At 24 h post-infection, the levels of unintegrated and integrated viral DNA were measured by fractionation of total cellular DNA into high molecular mass DNA (representative of integrated DNA) and low molecular mass DNA (representative of unintegrated DNA) by agarose gel electrophoresis followed by amplification of HIV-1 DNA as described by Pang et al. (1990) and Bukrinsky et al. (1991). As
The nature of the defect preventing integration of asymptomatic isolates is still under investigation. Our experiments do not distinguish whether the defect is at the step prior to or during the actual integration step. Since circular HIV-1 DNA with 1 or 2LTR is found only in the nucleus, its presence can be used as a convenient marker for nuclear transport of proviral DNA. The absence of circular DNA with 2LTR in H9 cells infected with asymptomatic isolates suggests that these isolates may have a defect in or prior to nuclear translocation. However, an alternative interpretation could be that the proviral DNA is transported to the nucleus by some amount of total HIV-1 DNA and 2LTR circles were measured by SK38/39 amplification of the gag gene and by M667/M884 primer pair amplification of the region spanning the 2LTR junction, respectively. H9 cells were infected with two asymptomatic isolates (1 and 2) and two AIDS isolates (3 and 4) with equal PCID. At 24 and 48 h post-infection, circular proviral DNA with 2LTR was found in cells infected with the two AIDS isolates, but not with the two asymptomatic isolates (Fig. 5). The level of gag DNA in cells infected with the asymptomatic isolates is comparable to that in cells infected with AIDS isolates. These results suggest that infectious asymptomatic isolates undergo normal virus entry and DNA synthesis, but fail at or before the integration step.

Discussion

A number of studies have reported that the determinant(s) of restriction of asymptomatic isolates in H9 cells at the virus entry level is located in the envelope protein, probably as a result of the envelope proteins being inaccessible to cellular receptors. This conclusion is supported by the fact that the envelope proteins of asymptomatic isolates are more resistant to neutralization by soluble CD4 and neutralization antibodies than the envelope proteins of AIDS isolates (Sullivan et al., 1995; Moore, 1995). In this study, we found that for asymptomatic isolates, in spite of restriction at the virus entry level a proportion of the virus population did enter the H9 cells and proceeded to replicate. However, the replication was restricted before or during nuclear translocation of newly synthesized DNA since this viral DNA was present only in the nonchromosomal low molecular mass fraction of cellular DNA and no circular HIV-1 DNA with 2LTR was detected in infected cells. A defect in nuclear translocation would prevent integration of the viral genome of asymptomatic isolates. Since integration of proviral DNA is essential for the expression of viral RNA and subsequent viral proteins (Sakai et al., 1993), a defect in integration could explain why asymptomatic isolates do not grow in H9 cells.
other mechanisms such as via a subviral particle, but neither integrates nor forms a 2LTR circle.

MA and Vpr proteins are implicated in nuclear transport of the preintegration complex in non-dividing cells (Bukrinsky et al., 1993; Heinzinger et al., 1994). These HIV-1 accessory proteins are not involved in nuclear transport of proviral DNA in dividing T cell lines such as H9 cells. However, this does not rule out the possible involvement of some other portion of the gag gene. A report by Nagy et al. (1994) suggested that the HIV protease may also be involved in the early phase of replication. The asymptomatic isolates reported here may have a defect in the protease gene which inhibits the formation of the cDNA-protein preintegration complex and thereby blocks the entry of proviral DNA into the nucleus. This could also explain the disappearance of gag DNA 4–8 days post-infection and its inability to integrate.

The presence of HIV-1 DNA in the infectious HIV-1 virion has been reported (Zhang et al., 1993; Lori et al., 1992; Trono, 1992). However, it is unlikely that the HIV-1 gag DNA observed 24 h post-infection with asymptomatic isolates was present in the input virions, for the following reasons: (a) H9 cells harvested 1 h after infection contained no detectable HIV-1 DNA (<1 copy) but contained a considerable level of HIV-1 DNA 24 h post-infection; (b) infection with heat-killed HIV did not produce any detectable HIV; (c) in an experiment with two asymptomatic and two AIDS isolates, synthesis of gag DNA by all four isolates was found to be equally sensitive to AZT (data not shown).

We have recently examined viral DNA synthesis following infection of PHA-stimulated PBMC with one of the asymptomatic and one of the AIDS isolates used in this study. Preliminary data indicate that the levels of integrated viral DNA in PBMC are also lower for the asymptomatic isolate compared to the AIDS isolate. Further studies are planned to determine which HIV-1 gene may be responsible for the inability of asymptomatic isolates to replicate by constructing various infectious molecular recombinants from molecular clones of an asymptomatic isolate and an AIDS isolate.

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References


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