Lack of human immunodeficiency virus type 1 (HIV-1) replication and accumulation of viral DNA in HIV-1-infected T cells blocked in cell replication

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Human immunodeficiency virus type 1 (HIV-1) infection of the CD4+ SupT and CEM cell lines, blocked in cell replication by the polymerase α inhibitor aphidicolin (APC), was studied. The APC-treated cells showed a lack of viral production, but the presence of single cell killing. High levels of unintegrated viral DNA forms were found in the infected APC-treated cells as compared with untreated cells. Moreover, an increased rate of viral replication occurred in the remaining viable cells following removal of APC. The results indicate that HIV-1 entry and reverse transcription can take place in cells blocked in the S phase of the cell cycle. Replication of infectious progeny virions appears to require de novo cell division. Finally, accumulation of viral DNA in cells during APC treatment can result in cytopathological effects and subsequent enhancement of virus production.

Introduction

Retrovirus infection of cells with production of progeny virus requires cellular DNA synthesis (Hsu & Taylor, 1982; Humphries et al., 1981; Richten et al., 1984; Springett et al., 1989; Varmus et al., 1977; Yang et al., 1980). The human immunodeficiency virus (HIV) has been reported to productively infect activated T lymphocytes that are undergoing cell division (Gowda et al., 1989). We investigated whether cellular replication was necessary for virus infection and whether HIV-1 could remain present in T cells kept in a quiescent state. Aphidicolin (APC) blocks DNA synthesis by inhibiting DNA polymerase α and has been used for dissecting molecular events during infection with avian and murine retroviruses (Hsu & Taylor, 1982; Springett et al., 1989). Our results with this compound indicate that in T cells blocked in cell division, HIV-1 can undergo reverse transcription with accumulation of high levels of unintegrated viral DNA species. This latter phenomenon was associated with single cell death of the non-dividing cells. When APC was removed from the culture medium, active virus replication in the remaining viable cells took place accompanied by destruction of the infected cells. This production of HIV-1, following removal of APC, yielded higher titres of progeny virus in comparison to infected cells that had not previously been treated with the compound.

Methods

Virus and virus assays. The HIV-1_{SF2} and HIV-1_{AF2} strains used in these studies were isolated in our laboratory from infected individuals (Levy et al., 1984; Levy & Shimabukuro, 1985). They were grown in human peripheral blood mononuclear cells and used in cell culture studies at 10^6 c.p.m./ml of reverse transcriptase (RT) activity. Replicating virus and viral protein expression were detected in the cell culture supernatant by assays for particle-associated RT activity (Hoffman et al., 1985), the p25 core antigen (ELISA, Du Pont), indirect immunofluorescence (Kaminsky et al., 1983) and the MT-4 plaque assay (Tateno & Levy, 1988).

Cell culture. The SupT cell line, obtained from James Hoxie, the CEM cell line (ATTC) and MT-4 cells (provided by N. Yamamoto, Tokyo, Japan) were grown in suspension culture in RPMI 1640 medium supplemented with 10% heated (56 °C, 30 min) foetal calf serum, 2 mM-glutamine and 1% antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin). For virus inoculation, the medium also contained polybrene (1 µg/ml). Cellular metabolic syntheses were monitored by incorporation of [³H]thymidine, -uridine and -leucine using the procedure of Nilsen & Baglioni (1979). APC was purchased from Sigma and suspended in DMSO at a stock concentration of 10 mg/ml. For each experiment, dilutions from this preparation were freshly made in the RPMI 1640 medium.

Virus infection. T cells were pretreated with APC for different time periods to stop cellular DNA synthesis, then infected with virus. After trypsinization to remove residual virus (Tang & Levy, 1991), cells were maintained in culture in the presence of the compound. The cells were tested for virus production before and after removal of APC by the assays listed above. The viability of infected cells was measured by trypan blue dye exclusion. Control untreated cells were infected and followed by the same procedures.

Analysis of cell cycle kinetics. Cell cycle progression was measured by flow cytometry using the staining technique for detergent-isolated

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nuclei as described previously (Krishan, 1975). Cells were washed in PBS and after ribonuclease treatment (200 units/ml) were stained for DNA content with propidium iodide (0.1 mg/ml and 0.6% NP40). Disaggregated nuclei were analysed on a FACScan flow cytometer, and cellular DNA content was adjusted and controlled with DNA QC particles and cellFIT software (Becton-Dickinson).

**Analysis of DNA.** The low M, DNA was prepared from cells extracted by a modified Hirt procedure (Chinsy & Soerio, 1981). In brief, the cells were washed in cold PBS pH 7-4, suspended in 10 mM-Tris-HCl pH 7-5, 10 mM-EDTA, 150 mM-NaCl and lysed in 10% SDS, then heated at 65 °C for 20 min. NaCl was added to 1 M and the lysates were kept at 4 °C overnight. Samples were centrifuged at 12000 r.p.m. in a Sorvall HB-4 rotor for 30 min. The Hirt supernatants were digested with proteinase K to a final concentration of 400 μg/ml for 1 h at 37 °C, extracted with phenol–chloroform and precipitated with ethanol.

The high M, DNA was prepared from cellular DNA by standard procedures (Pellicer et al., 1984). Cells were washed with PBS and disrupted by 0.5% SDS, then digested with proteinase K (100 μg/ml) for 12 h at 37 °C. The viscous lysates were extracted by phenol. DNA was collected by spooling onto a glass rod after the addition of 3 volumes of ethanol to the aqueous phase. Recovered DNA was digested with RNase and re-extracted with phenol–chloroform, then precipitated with ethanol. High M, DNA was digested with the restriction enzyme SacI (Sigma) according to the manufacturer's recommendation. These DNAs were probed by Southern blotting with a 32P-labelled nick-translated HIV-1-specific whole genomic probe, pZ6 (Luciw et al., 1984) provided by P. Luciw, Davis, Ca., U.S.A. Lambda DNA HindIII fragments served as size markers.

**Results**

**Effect of APC on DNA synthesis and cell cycle in the SupT cell line**

To evaluate the role of cellular DNA synthesis in HIV-1 infection, we chose the compound APC which is known to block cellular DNA polymerase α (Huberman, 1981; Pedrali-Noy et al., 1980). At 4 μg/ml, APC had substantial effects on DNA synthesis by SupT cells and gave nearly 100% inhibition without disturbing cell

### Table 1. HIV expression in infected T cells in the presence or absence of APC

<table>
<thead>
<tr>
<th>Cell line†</th>
<th>HIV-1 expression*</th>
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<tbody>
<tr>
<td></td>
<td>RT (× 10⁻³ c.p.m./ml)</td>
</tr>
<tr>
<td><strong>SupT</strong></td>
<td></td>
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<tr>
<td>In the absence of APC (control)</td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>1.4</td>
</tr>
<tr>
<td>Day 3</td>
<td>2.1</td>
</tr>
<tr>
<td>Day 6</td>
<td>5.6</td>
</tr>
<tr>
<td>Day 8</td>
<td>13.4</td>
</tr>
<tr>
<td>In the presence of APC</td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>1.0</td>
</tr>
<tr>
<td>Day 3</td>
<td>1.7</td>
</tr>
<tr>
<td>After removal of APC</td>
<td></td>
</tr>
<tr>
<td>Day 6</td>
<td>25.2</td>
</tr>
<tr>
<td>Day 8</td>
<td>123.5</td>
</tr>
<tr>
<td><strong>CEM</strong></td>
<td></td>
</tr>
<tr>
<td>In the absence of APC (control)</td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>1.0</td>
</tr>
<tr>
<td>Day 3</td>
<td>4.5</td>
</tr>
<tr>
<td>Day 6</td>
<td>56.7</td>
</tr>
<tr>
<td>Day 8</td>
<td>202-0</td>
</tr>
<tr>
<td>In the presence of APC</td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>0.7</td>
</tr>
<tr>
<td>Day 3</td>
<td>2.7</td>
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<tr>
<td>After removal of APC</td>
<td></td>
</tr>
<tr>
<td>Day 6</td>
<td>155-6</td>
</tr>
<tr>
<td>Day 8</td>
<td>440-4</td>
</tr>
</tbody>
</table>

* HIV-1 expression was determined by RT activity in the culture fluid, p25 core antigen production, viral protein expression measured by indirect immunofluorescence (IFA) and infectious virus particle production as determined by the MT-4 plaque assay (Tateno & Levy, 1988). HIV-1sf33-infected cells without APC treatment were used as controls.

† The CD4+ T cell lines, SupT and CEM, were pretreated with APC (4 μg/ml) for 24 h. The cells were infected with HIV-1sf33 for 1 h in the presence of polybrene (1 μg/ml). Residual virus on the cell surface was removed by trypsin treatment (Tang & Levy, 1991). These HIV-1sf33-infected APC-treated cells were then cultured in the presence of APC for 3 days. After 3 days, the cells were washed and cultured in fresh complete medium without APC for a further 5 days.

‡ Time after initial virus inoculation. A representative of at least two separate experiments is presented.
HIV-1 infection of non-dividing cells

Fig. 1. Effect of APC on cellular metabolism and the cell cycle. (a) Effect of APC on RNA (○), DNA (●) and protein (△) synthesis in SupT cells. The cells were treated with APC at the indicated concentrations for 18 h. Subsequently, the labelled nucleotide or amino acid was added for 6 h to the cells kept in the continual presence of APC. All results are expressed as percentages of inhibition compared to the data with control untreated SupT cells. A representative of two separate experiments is presented. The control values (mean ± S.D.) were 30240 ± 517 c.p.m. for DNA, 12924 ± 1035 c.p.m. for RNA and 150048 ± 25741 c.p.m. for protein. (b) Effect of APC on cell cycle kinetics. SupT cells were (i) untreated or (ii) treated with 4 μg/ml of aphidicolin for 24 h. The APC-treated cells were then washed and placed in fresh complete medium for 24 h (iii). A total of 10^6 cells was analysed for each histogram. The results indicate a block at the S phase in cells under APC treatment, and a return to a normal cycle after removal of the APC.

Effect of APC on HIV-1 infection of SupT cells

In evaluating HIV infection of cells blocked in DNA synthesis, SupT or CEM cells were pretreated with APC (4 μg/ml) for 24 h. Subsequently, either HIV-1SF2 or HIV-1SF33 was inoculated into the cultures. After HIV inoculation, APC was left in the culture fluid for various time periods. First, the viability of APC-treated infected and uninfected cells was compared to that of the untreated infected and uninfected cells. The data indicated that, after more than 3 days post-infection, greater than 50% of infected cells continually exposed to APC underwent single cell killing (Fig. 2, 3 b). Syncytium formation was not observed. This cytopathology did not result from toxicity of the compound nor from HIV-1 infection alone since control uninfected cells exposed to the same concentration of APC as well as cells infected with the virus alone were not so extensively lysed in this time period (Fig. 2 and 3).
Expression of virus was measured in SupT and CEM cells infected with HIV-1SF33 (Table 1). These studies indicated that cells treated with APC and acutely infected with HIV-1SF33 did not express substantial levels of viral proteins as detected by the immunofluorescence assay nor produce any progeny virus as measured by RT activity or plaque-inducing particles in the culture medium even though many cells underwent cell killing after HIV-1 infection. Small amounts of viral core antigen (p25) were detected, indicating only low level expression of this viral protein.

Within 3 to 5 days after removal of the compound, an increased peak of virus production was observed in the surviving viable T cells (Fig. 4). This replication of HIV, after removal of APC, was greatly enhanced over that observed with the infected SupT or CEM cells not treated with APC. The increase in HIV-1 production was accompanied by substantial changes in cytopathology in the SupT cells, characterized by cell aggregation and balloon degeneration (Fig. 3d). This enhancement in virus replication was confirmed by virus titration in MT-4 cells; high levels of infectious virus were observed (Table 1). A similar enhanced production of HIV-1SF33 occurred in CEM cells (Table 1) but it did not lead to formation of multinucleated cells. CEM cells therefore, maintained this biological characteristic even during substantial HIV-1 replication (Tang & Levy, 1991). Finally, when HIV-1SF33 chronically infected SupT cells were treated with APC for 48 h, cell division was arrested and HIV-1 production was decreased by 25%. Cell viability was unchanged. After removal of APC, virus production reached levels of the control cultures in 3 days. In this case, in which virus integration in all cells had already previously occurred, no enhanced replication of virus was observed (data not shown).

Effect of APC treatment on the HIV-1 replicative process

To determine the molecular events associated with this block in HIV-1 replication and onset of single cell killing, the viral DNA was analysed after different time periods. The CD4+ SupT and CEM cells were pretreated with the non-toxic concentration of APC (4 µg/ml) for 24 h. The treated cells were then infected with HIV-1SF33 and maintained in the presence of APC. Other SupT cells were infected with virus and left untreated. The
appearance of HIV-1 DNA forms was measured at day 2 after viral inoculation. HIV-infected cells were extracted by the Hirt procedure to isolate low $M_I$ DNA. The DNA was examined for HIV-1 sequences by Southern blot hybridization.

In HIV-1-infected control SupT or CEM cells, unintegrated viral DNA bands at 6.5, 9.4 and approximately 23 kb were observed. In these untreated cells, the linear forms were assumed to be located at 9.4 kb, whereas the 6.5 kb DNA most probably represented circular forms (Fig. 5a, lanes 1 and 3). In infected cells treated with APC, the unintegrated viral DNA forms were observed only in a broad smear in the range of about 9.0 kb (Fig. 5a, lanes 2 and 4). No discrete bands suggestive of circular forms were detected. This unintegrated viral DNA could represent somewhat degraded forms of viral DNA caused by the death of APC-treated cells. The 23 kb DNA in all cell cultures was considered to be a multimeric form of single DNA strands (Masuda & Harada, 1990). The results using densitometry also indicated a 10- to 20-fold greater amount of unintegrated DNA accumulating in APC-treated infected SupT and CEM cells compared to control infected cells (data not shown).

The high $M_I$ DNA was analysed to examine the extent of HIV integration occurring during and after APC treatment. Following standard extraction procedures and digestion with $SacI$, DNA fragments at 5.5 and 3.8 kb (Fig. 5b) were detected in the infected cells treated or not treated with APC (Fig. 5b, lanes 2 and 3). This DNA pattern was consistent with results obtained with high $M_I$ DNA from HIV-1SF33 chronically infected HUT 78 cells (Fig. 5b, lane 1). Similar bands of DNA
were observed in the cells removed from APC (Fig. 5, lane 5), but the amount was about 100-fold more abundant (by densitometry) than that in the control untreated cells (Fig. 5, lane 4). The results suggest that in the presence of APC some integration of HIV-1 occurs. After removal of the drug, the accumulated unintegrated viral DNA leads to efficient viral integration and enhanced progeny virus production.

Discussion

These studies indicate, using established cell lines and APC, an inhibitor of eukaryotic DNA polymerase \( \alpha \), that HIV-1 can infect cells arrested in cell division. Unintegrated DNA forms accumulate in the infected cell at a high level. Viral DNA persists without evidence of infectious virus replication in the cell until subsequent removal of the inhibitor. The data from molecular studies demonstrate that with APC reverse transcription takes place and unintegrated HIV-1 DNA forms increase during the time of APC treatment and are maintained for long periods of time extrachromosomally. Thus, the results indicate, in contrast to conclusions by others, that accumulation of unintegrated DNA can occur in cells without the need for infectious virus production and re-infection (Besanký et al., 1991; Shaw et al., 1985).

In concert with other observations, the large quantity of unintegrated viral DNA without evidence of substantial viral protein expression in APC-treated cells correlated directly with cytopathic effects and single cell killing. In cells infected with the spleen necrosis virus, an accumulation of unintegrated viral DNA is also linked to cell killing (Keshet & Temin, 1979). High levels of unintegrated viral DNA have also been associated with infection by other lentiviruses such as visna virus (Brahic & Haase, 1978; Haase et al., 1977). Studies with avian leukaemia viruses, reticuloendotheliosis viruses in vitro and some strains of feline leukaemia viruses both in vitro and in infected cats also demonstrate a close correlation between cytopathic effects of the virus and presence of high levels of unintegrated viral DNA (Keshet & Temin, 1979; Mullins et al., 1986). Likewise, the cytopathic effects of HIV-1 in some studies have been coupled with accumulation of unintegrated forms (Besanký, 1991; Levy et al., 1985; Pauza & Galindo, 1989; Robinson & Zinkus, 1990; Shaw et al., 1984). Nevertheless, whether the increased levels of unintegrated viral DNA or the enhanced production of an unknown cellular protein is responsible for the cell death has not been firmly established.

The unintegrated viral DNA in cells treated with APC was also associated with an increase in titres of replicating virus in the surviving cells following removal of the compound. This observation suggests that the accumulated DNA species once released from the inhibitory effects of APC can lead to virus production. However, whether virus replicates from the unintegrated or integrated state could not be answered directly by these experiments.

These studies were aimed at assessing the importance of cellular DNA synthesis on HIV-1 replication. They represent an alternative in vitro model for virus infection of non-dividing cells such as macrophages and microglial cells in the brain (Price et al., 1988; Shaw et al., 1985; Watkins et al., 1990; Wiley et al., 1986). Some integration of HIV-1 did appear to take place in these cells blocked by APC in the S phase. Similarly, HIV might integrate into non-dividing macrophages in the S phase and result in the virus replication observed with these cells. This possibility is under study. Finally, the association of high levels of unintegrated viral DNA with cell death could have clinical relevance. Autopsied brains of AIDS patients have also shown a large amount of unintegrated HIV DNA (Pang et al., 1990). This phenomenon could be contributing to the neurological disease observed.

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