The cytopathic effect of human immunodeficiency virus is independent of high levels of unintegrated viral DNA accumulated in response to superinfection of cells

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Large quantities of genome-sized viral DNA are detected in the nucleoplasm of CD4+ T cells infected with human immunodeficiency virus type 1 (HIV-1). This unintegrated HIV DNA is in the form of both circular and linear species. Accumulation of such DNA occurs gradually during a 5 day HIV infection and is correlated with the proportion of cells involved in the production of HIV proteins. To pinpoint the stage in a synchronized HIV infection during which accumulation of HIV DNA occurs, high titres of HIV were employed to infect CEM cells to infect the majority of cells by the input virus. By this latter infection, more than 95% of cells became producers of HIV proteins at 48 h post-infection (p.i.) concomitantly with the development of the c.p.e, of HIV, manifested by formation of syncytia and induction of cell death by apoptosis. Addition of azidothymidine (AZT) or neutralizing anti-gp120 monoclonal antibodies at 8 h p.i. did not alter the course of virus infection nor the amount of virus produced at 48 h p.i. but the accumulation of unintegrated HIV DNA was drastically reduced. These results indicate that viral DNA accumulates as a result of superinfection of cells late in the virus cycle. The development of the c.p.e, of HIV was inhibited in the presence of neutralizing antibodies, whereas in the presence of AZT the accumulation of unintegrated HIV DNA was completely blocked without apparent effect on the c.p.e. These observations indicate that the c.p.e, of the HIV infection, which is manifested by syncytium formation and apoptosis, does not require superinfection of cells or accumulation of unintegrated viral DNA.

Introduction

Human immunodeficiency virus (HIV) is considered to be the aetiological agent of AIDS in which CD4+ T lymphocytes progressively become depleted (Rosenberg & Fauci, 1991). The precise mechanisms responsible for the depletion of CD4+ T lymphocytes is not yet clear. Recently, programmed cell death or apoptosis has been suggested to be responsible, at least in part, for T4 lymphocyte depletion in HIV-seropositive individuals (Ameisen & Capron, 1991; Ameisen, 1992). Viral infection may play a major role in the immunopathogenesis of HIV infection. In favour of this latter explanation are a number of observations indicating that disease progression is correlated with the emergence of virus variants characterized by increasing cytopathic phenotypes (Cheng-Mayer et al., 1988; Fenyö et al., 1988; Tersmette et al., 1988; Schellekens et al., 1992) and the close correlation between viral load, decreasing CD4+ T cell count and disease progression (Schnittmann et al., 1990; Hsia & Spector, 1991; Bagasra et al., 1992).

HIV infects lymphocytes, monocytes and macrophages by binding to its principal receptor, the CD4 molecule (Marsh & Dalgleish, 1988). After viral entry, HIV RNA is reverse-transcribed in the cytoplasm by the viral transcriptase to produce the proviral DNA, which is then integrated into the host chromosomal DNA (for a recent review, see Cullen, 1991). In general, HIV infection of cell cultures results in the generation of an acute and/or chronic infection. In both cases, virus is produced and becomes released by budding at the cellular membrane. Acute infection is characterized by a typical c.p.e, manifested by ballooning of cells, formation of syncytia and subsequently by cell death. On the other hand, chronically infected cells do not show this typical c.p.e, despite their constant capacity to produce infectious HIV particles. This difference between acute and chronic infection has been reported to be due in part to a lower amount of virus particles produced and down-regulation of CD4 receptors in persistently infected cells (Stevenson et al., 1988). Recently, our group and others have suggested that the c.p.e, of HIV-1 and HIV-2 is associated with apoptosis in CD4+ cell cultures (Laurent-Crawford et al., 1991; Terai et al., 1991). Apoptosis is a
Physiological cell suicide mechanism occurring in metabolically active cells and is manifested by the activation of a Ca²⁺-dependent nuclear DNAse responsible for the cleavage of chromatin at nucleosomal junctions (Duvall & Wyllie, 1986). Accordingly, a classical DNA fragmentation was observed in CEM cells during acute infection. In contrast, syncytium formation and DNA fragmentation did not occur in chronically infected CEM cells (Laurent-Crawford et al., 1991).

Previously, the c.p.e. of HIV in cell cultures has been correlated with the accumulation of unintegrated viral DNA occurring late in the course of infection (Shaw et al., 1984; Muesing et al., 1985). This is analogous with other retroviral systems, including avian viruses (Keshet & Temin, 1979; Weller et al., 1980), feline leukaemia viruses (Mullins et al., 1986), visna viruses (Haase, 1986) and primate foamy viruses (Mergia & Luciw, 1991) in which cell death has been associated with the accumulation of unintegrated retroviral DNA as a result of massive second-round superinfection (Weller et al., 1980). In the case of HIV infection, it has also been observed that accumulation of unintegrated viral DNA is due to superinfection (Robinson & Zinkus, 1990; Pauza et al., 1990). These DNA species are in three major forms: a linear copy of the viral genome with two long terminal repeat (LTR) sequences and two circular DNA species with one or two LTRs (Varmus & Swanstrom, 1984). Accumulation of unintegrated HIV DNA has also been observed in vivo in lymph node tissues from AIDS patients (Shaw et al., 1984). Moreover, owing to very sensitive detection methods now available, it has been shown that high levels of unintegrated HIV DNA can be detected in the brain of AIDS dementia patients (Pang et al., 1990) as well as in quiescent T lymphocytes from asymptomatic individuals (Bukrinsky et al., 1991). A recent study in macaques infected with simian immunodeficiency virus has indicated that much of the viral DNA is unintegrated in most tissues taken at the time of autopsy (Hirsch et al., 1991).

The role of unintegrated viral DNA in the evolution of AIDS in HIV-infected patients remains to be investigated. On the other hand, by using a synchronous HIV infection of cell cultures, we further confirm that the accumulation of unintegrated viral DNA is due to superinfection of cells at later stages of virus infection. In addition, we demonstrate that this unintegrated HIV DNA is not essential for the typically observed c.p.e. of HIV.

Methods

HIV and cells. The HIV-1 Lai isolate previously referred to as HIV-1 Bru (Wain-Hobson et al., 1991) was used in this study to infect CEM clone 13 cells which are derived from the human lymphoblastoid cell line CEM (ATCC-CCL 119) that expresses the T4 antigen to a high level. Cells were cultured in suspension medium RPMI-1640 (Gibco-BRL) containing 10% (v/v) fetal calf serum and 2 μg/ml polybrene (Sigma).

Acute synchronous infection of CEM cells. An HIV-1 stock with a high infectivity was prepared by several consecutive passages of the original HIV-1 Lai isolate on CEM cells (Laurent et al., 1990). The titre of the viral stock was evaluated using CEM cells according to the standard dilution procedure. In all infections, cells (5 × 10⁶/ml) were incubated with virus for 1 h at 37°C before centrifugation and resuspension in fresh culture medium at 1 × 10⁶ cells/ml. Virus stocks that gave a time course of infection lasting not more than 3 days were considered appropriate for a synchronous infection of all cells in the culture, referred to as 1 synchronous infectious dose (containing 400 ng of HIV particle-associated p25 per 10⁷ cells). In such an infection, more than 90% of cells become producers of HIV proteins (according to an immunoenzymatic assay; Laurent et al., 1989) at 48 h post-infection (p.i.) at which time maximal c.p.e., manifested by ballooning of cells and syncytium formation, is observed. Evidence that most of the cells become infected by the input virus was provided by the observation that infection is not modified in the presence of azidothymidine (AZT) when added at 6 h p.i. In such a synchronous infection, cell lysis occurs at day 3 p.i.

Monoclonal antibodies (MAbs). MAb N11/20 specific for gp120 was the generous gift of F. Nato of Hybridolaboratory in the Institut Pasteur, Paris. This antibody recognizes an epitope in the V3 loop of gp120 (F. Traincard, unpublished observations). MAb 110/4 specific to the V3 loop of gp120 and MAb 25/3 specific to p25 were obtained from Genetic Systems.

Preparation of cell extracts. For the recovery of cytoplasm separately from nucleoplasm, cells were disrupted in cytoplasm extraction buffer containing 20 mM-Tris·HCl pH 7.6, 0.15 M NaCl, 5 mM-MgCl₂, 0.2 mM-PMSF, 100 units/ml aprotinin and 0.5% Triton X-100. After centrifugation at 15000 g, the supernatant contained the cytoplasm whereas the intact nuclei were pelleted. The nuclei were then extracted in lysis buffer containing 10 mM-Tris·HCl pH 7.6, 0.4 M NaCl, 1 mM-EDTA, 0.2 mM-PMSF, 100 units/ml aprotinin (Iniprol) and 1% Triton X-100. This nuclear extract was then centrifuged at 12000 g to separate the nucleoplasm from the high Mr chromatin.

Analysis of unintegrated HIV DNA. The nucleoplasm (prepared as described above) contained the low Mr DNA (cellular and unintegrated HIV DNA) whereas the pellet of the nuclear extracts contained the high Mr chromatin. Nucleoplasm was incubated with 20 μg/ml RNase A for 1 h at room temperature, then 100 μg/ml proteinase K for 2 h at 37 °C, followed by extraction with phenol–chloroform–isoamyl alcohol (25:24:1) and precipitation with ethanol. The pellet was analysed for the presence of unintegrated HIV-1 DNA by slot blot and Southern blot assays. The 32P-radio labelled HIV DNA probe corresponding to the entire HIV-1 genome was from p-Bru 2, a molecular clone of HIV-1 Lai provided by K. Peden.

Analysis of histones. The c.p.e. of HIV-1 Lai manifested by syncytium formation is associated with apoptosis (Laurent-Crawford et al., 1991). Accordingly, the nucleoplasm of infected cells contains nucleosomes which become accumulated as a consequence of chromatin fragmentation during apoptosis. The presence of nucleosomes could be monitored either by extraction of the low Mr DNA to analyse the oligonucleosomal DNA fragments or by the recovery of histones (H2A, H2B, H3 and H4) after solubilization in a non-ionic detergent, such as SDS. For this latter purpose, crude nucleoplasm extracts were diluted onefold in electrophoresis sample buffer (0.125 M-Tris·HCl pH 6.8, 2% w/v SDS, 20% glycerol and 1% 2-mercaptoethanol) and boiled 5 min before analysis by polyacrylamide gel (15%) electrophoresis in SDS. Histones were clearly detectable by staining the gel
**Cytopathic effect of HIV infection**

(a) The proportion of cells expressing viral proteins. The kinetics of infection was monitored by an immunoenzymatic assay (Laurent et al., 1989) using MAbs 25/3 and 110/4 to reveal the major core protein p25 and the external envelope glycoprotein gp120, respectively. The ordinate gives the proportion (% positive cells) of cells expressing p25 (●) and gp120 (■).

(b) Slot blot assay. At different days p.i., cells were analysed for the accumulation of unintegrated HIV-1 DNA in the nucleoplasm (Methods). Material corresponding to 2 x 10⁶ cells was used at days 1 to 5 p.i.

(c) Southern blot assay. Unintegrated HIV DNA was extracted from the nucleoplasm of HIV-1-infected cells 5 days p.i. (Methods). DNA without (lane 1) or with (lane 2) BamHI digestion was suspended in electrophoresis sample buffer (10 mM-Tris-HCl pH 8.0, 1 mM-EDTA) and analysed by electrophoresis on a 1% agarose gel containing 0.5 μg/ml ethidium bromide at 80 V for 5 h. On the right is the position of DNA markers. On the left NC, L and C indicate the position of the nicked circular, linear and circular forms of the HIV-1 DNA, respectively. In sections (b) and (c), a 32P-labelled probe corresponding to the entire HIV-1 genome was used. Autoradiographs are shown.

**Results**

_A multiple cycle kinetics in cells infected with HIV at low multiplicity_

All the experiments were carried out with a stock of HIV-1 Lai (Wain-Hobson et al., 1991; previously referred to as HIV-1 Bru) prepared on CEM cells (Laurent et al., 1990). Routinely, after 1 h of incubation, the input virus was removed and cells were suspended in fresh culture medium. At a dose of HIV corresponding to 20 ng of particle-associated p25 per 10⁶ cells, the first signs of the c.p.e. were clearly observed 2 to 3 days p.i. by vacuolization of cells and appearance of syncytia. Fig. 1 gives a typical kinetics of infection showing the proportion of cells involved in the synthesis of viral proteins, and the accumulation of unintegrated HIV-1 DNA. MAbs specific for the HIV-1 envelope glycoprotein gp120 and the major core protein p25 were employed here in an immunoenzymatic staining assay to estimate the proportion of HIV-producing cells. During the course of infection, the proportion of cells producing HIV proteins gradually increased reaching 82 and 95% for gp120 and p25, respectively, on day 5 p.i. The difference between the detection of gp120 and p25 is due probably to the sensitivity of the assay for each protein. Consequently, the number of HIV-producing cells at 5 days p.i. could be considered to be more than 90%, during which time only a very small proportion of infected cells remained as single cells. At this stage, syncytium formation was maximal and was associated with apoptosis as we have reported previously (Laurent-Crawford et al., 1991). Infected cells became lysed on day 6 p.i.

Accumulation of unintegrated HIV-1 DNA in the infected cells was also gradual. In fact very little HIV DNA was observed in the nucleoplasm of infected cells 1 to 2 days p.i. However, from day 3 onwards there was a dramatic increase which was highly correlated with the proportion of HIV-producing cells (Fig. 1). A possible explanation for the gradual increase in the proportion of HIV-producing cells is the fact that only a small number of cells become infected by the input virus. Accordingly, only a very small proportion of cells were found to be producers of HIV proteins on day 1 p.i.
Table 1. AZT added 6 h.p.i. during multiple cycles of HIV infection reduces the proportion of infected cells and virus production

<table>
<thead>
<tr>
<th>Agent</th>
<th>Time of addition (h)</th>
<th>Positive cells (%) on day 4</th>
<th>Virus production on day 5 [p25 (ng/ml)]</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>55</td>
<td>1158</td>
<td></td>
</tr>
<tr>
<td>AZT</td>
<td>6</td>
<td>5</td>
<td>176 (85)</td>
<td></td>
</tr>
<tr>
<td>AZT</td>
<td>24</td>
<td>12</td>
<td>258 (78)</td>
<td></td>
</tr>
<tr>
<td>AZT</td>
<td>48</td>
<td>25</td>
<td>548 (53)</td>
<td></td>
</tr>
</tbody>
</table>

* CEM cells were infected with HIV-1 as in the legend of Fig. 1. In separate but similarly infected cultures, AZT (5 μM) was added at 6, 24 and 48 h.p.i. On day 4, the proportion of HIV protein-expressing cells was monitored by an immunoenzymatic assay using MAb 25/3 specific for p25 (Laurent et al., 1989). Without AZT, the proportion of cells positive for HIV proteins was 55 and 95% on days 4 and 5, respectively. Viral production was measured on day 5 p.i. by assaying the concentration (ng/ml) of particle-associated p25 in the culture supernatant (percentage inhibition is given in parentheses).

![Fig. 2. Synthesis of HIV-1 proteins during the course of a synchronous infection of CEM cells. CEM cells were infected with 1 synchronous infectious dose of HIV-1 (Methods: culture supernatant containing 400 ng of particle-associated p25 per 10⁶ cells) and aliquots of such infected cells (5 × 10⁶) were analysed for the synthesis of viral proteins by metabolic labelling with [35S]methionine at various times: 16 to 24 h (lanes 1), 24 to 32 h (lanes 2), 48 to 56 h (lanes 3) and 72 to 80 h (lanes 4). Extracts from cells (a) and the virus pellets (b) were then immunoprecipitated with an HIV-1-positive serum and analysed by SDS-PAGE and fluorography. For metabolic labelling, cells were incubated in MEM without [35S]methionine or serum, but supplemented with 200 μCi/ml of [35S]methionine (specific activity > 1000 Ci/mmol) for 8 h for each point (Laurent et al., 1989). The position of HIV-1 proteins is indicated on the left: the precursor of the envelope glycoprotein (gp160), the external (gp120) and transmembrane (gp41) envelope glycoproteins, the reverse transcriptase (p68), the gag precursor (p55) and its partially cleaved product (p40), and the major core protein (p25).]

1. Virus produced from the first round of infection might then infect uninfected cells. Thus, input infectious virus can be amplified by successive rounds of infections until all cells become infected. This is probably the case, since addition of poly(A).poly(U), an inhibitor of viral entry (Laurent-Crawford et al., 1992a), at day 1 p.i. blocked the second and successive rounds of infection (data not shown). Similarly, addition of AZT at days 1 and 2 p.i. resulted in the inhibition of viral production due to its inhibitory effect on the successive rounds of infection (Table 1).

Southern blotting analysis of the unintegrated HIV DNA accumulated at day 5 p.i. revealed three major forms migrating at positions corresponding to 6-0, 9-5 and approximately 25 kb (in some experiments migrating at positions 20 to 23 kb) (Fig. 1). The 9-5 kb DNA band corresponds to the linear full-length genome of HIV-1 whereas the 6-0 kb and the 25 kb bands should correspond to supercoiled and nicked circular species with one or two LTRs, in accord with previously published observations (Charneau & Clavel, 1991; Kim et al., 1989; Pauza et al., 1990). Digestion of this HIV-1 genome with BamHI at the unique site at position 8520 (Wain-Hobson et al., 1985) resulted in one broad band, consisting of the circular forms converted into linear forms of 9 and 9-5 kb and the large fragment of 8-6 kb cleaved from the linear species (Fig. 1).

Establishment of a synchronous infection of the majority of cells in a culture

The experiments described in Fig. 1 demonstrated that at a dose of HIV corresponding to 20 ng of p25 per 10⁶ cells, only a small proportion of cells become infected by the input virus. Thus investigating the relationship of the accumulation of unintegrated HIV DNA to the c.p.e. of HIV required the use of infection conditions in which a majority of cells become infected by the input virus. This was achieved arbitrarily by the use of higher doses of HIV that corresponded to 400 ng of particle-associated p25 per 10⁶ cells. This dose was referred to as 1 synchronous infectious dose. At this dose the course of infection was 3 days. In such infections, synthesis of HIV-1 proteins was detectable as soon as 16 h.p.i., reaching a maximal synthesis by 48 h.p.i. (Fig. 2) at a time when more than 90% of cells became producers of HIV proteins (detected by an immunoenzymatic assay). The c.p.e. of the HIV infection which was manifested by ballooning of cells and syncytium formation was observed at 48 h.p.i. Most of the unintegrated HIV DNA became accumulated between 24 and 48 h.p.i. (Fig. 3).

To determine the shortest time necessary for virus adsorption, entry and reverse transcription of HIV RNA to initiate a synchronous infection of almost all cells in the culture, AZT was added at different times after virus adsorption. As expected, addition of AZT with the virus or up to 2 h after virus adsorption resulted in the
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C.p.e. - - - + - -

p25-positive cells (%) 0 0 50 95 93 96

Anti-gp 120 MAb - - - + +

Time p.i. (h) 0 8 24 48 48 48

Fig. 3. Unintegrated HIV DNA accumulates as a consequence of superinfection of cells during a synchronous infection of CEM cultures. CEM cells were infected with 1 synchronous infectious dose of HIV-1 (as in the legend of Fig. 2) and analysed for the accumulation of unintegrated HIV DNA at 8, 24 and 48 h p.i. (as in Fig. 1c). An autoradiograph is shown. The c.p.e. was monitored by the formation of syncytia (indicated by plus symbol; minus symbol indicates no syncytia). The proportion of cells producing HIV-1 p25 was estimated using MAb 25/3 (as in Fig. 1a). MAbs 110/4 and N11/20 (at 10 μg/ml; the last two lanes, respectively) were added to the infected cultures at 8 h p.i. and the cultures were analysed at 48 h p.i. Both antibodies blocked the formation of syncytia and the accumulation of unintegrated HIV DNA but did not have any apparent effect on the proportion of p25-positive cells.

The effect of anti-gp120 neutralizing antibodies on the synchronous infection

The synchronous HIV infection of CEM cells was characterized by MAbs specific for gp120. MAbs 110/4 and N11/20, both recognizing the V3 loop on gp120 of HIV-1 LAI, completely neutralized HIV-1 LAI infection without significantly affecting the binding of gp120 to the CD4 receptor (Kinney-Thomas et al., 1988; Linsley et al., 1988). Under the synchronous infection conditions, addition of either antibody together with HIV resulted in the inhibition of virus infection as expected.

24 h p.i., resulted in a 78% reduction of virus production due to inhibition of infection of the remaining uninfected cells (Table 1).

Higher doses of HIV (corresponding to 400 ng of p25/10⁶ cells) were required here to achieve a synchronous infection of CEM cells, since a very small proportion of HIV particles become bound to CEM cells. In fact less than 2% of HIV particles are found to bind to CEM cells and, furthermore, only 50% of the bound virus enters cells (Krust et al., 1993), implying that the infectivity of the input virus is around 1%. Thus the virus stocks contain a high proportion of defective virus particles probably due to partial but continual leakage of gp120 from virus particles reducing their affinity to bind to CD4 receptor molecules.
Fig. 5. Inhibition of the c.p.e. of HIV-1 by anti-gpl20 antibody but not by AZT treatment. CEM cells under synchronous infection conditions (Fig. 2 to 4) were incubated with MAb 110/4 (10 μg/ml) or AZT (5 μM). Both agents were added at 8 h p.i. The c.p.e. was monitored under a light microscope by the formation of syncytia. (a) Uninfected CEM cells. (b) HIV-infected CEM cells. (c) HIV-infected cells plus anti-gpl20 at 8 h p.i. (d) HIV-infected cells plus AZT at 8 h p.i.

However addition of these antibodies at 8 h p.i. did not modify the course of infection nor did it affect the intensity of viral production. Fig. 4(b) shows the pattern and the quantity of HIV proteins found in cells at 48 h p.i. It should be noted that both antibodies were functional since they blocked the syncytium formation as well as ballooning of cells routinely observed at 48 h p.i. in cultures without the antibodies (Fig. 5). Furthermore, these antibodies almost completely blocked the accumulation of unintegrated HIV DNA (Fig. 3, lanes labelled + for MAb anti-gpl20). Therefore in this synchronous infection, accumulation of the unintegrated HIV DNA results from superinfection, as previously reported by others using longer times of infection (Robinson & Zinkus, 1990; Pauza et al., 1990; Besansky et al., 1991). Our results also indicate that superinfection is not necessary for optimal viral protein expression when the majority of cells become infected by the input virus.

**AZT added at 6 h p.i. blocks the accumulation of unintegrated viral DNA without modifying the c.p.e. of HIV**

During a synchronous infection, HIV RNA replication was blocked by the addition of AZT at different times following virus adsorption. At 48 h, virus infection was monitored by the development of c.p.e. (formation of syncytia), the accumulation of unintegrated HIV DNA and by the production of virus, estimated by the concentration of p25 in the culture medium (Fig. 6). In a previous study, we reported that the c.p.e. of HIV-1 Lai and HIV-2 ROD is associated with apoptosis in CD4 T cell cultures (Laurent-Crawford et al., 1991) and that this effect is mediated by the expression of HIV envelope glycoproteins (Laurent-Crawford et al., 1992b). One marker for apoptosis or programmed cell death is the presence of cellular low Mr DNA consisting of multimers of a nucleosome-length unit of 200 bp. As an alternative to this method, we have demonstrated that Coomassie blue staining of histones associated with these low Mr DNA fragments can be used as a convenient assay to monitor apoptosis in HIV-infected cells (Laurent-Crawford et al., 1991). Accordingly, the detection of histones was used to quantify the relative level of apoptosis occurring in these infected cultures (Fig. 6). Addition of AZT together with the virus, or 1 h after virus adsorption, resulted in a dramatic inhibition of virus production, development of syncytia and apoptosis. On the other hand, AZT added from 6 h onwards did not cause any apparent modification in the kinetics and the production of virus nor in the development of syncytia and apoptosis (Fig. 5 and 6). Treatment with AZT was effective, since accumulation of the unintegrated HIV
DNA was almost completely inhibited (Fig. 6, insert). These results confirm once again that an interval of a minimum of 6 h was required during which time the input viral RNA was reverse-transcribed into proviral DNA to initiate the synchronous infection. The fact that AZT added at 6 h p.i. did not modify the development of the c.p.e. but drastically inhibited the accumulation of unintegrated HIV DNA suggests that these two events are not related.

Single-cell killing by lysis in the presence of anti-V3 MAbs

Under well defined experimental conditions, the c.p.e. of HIV-1 and HIV-2 in CD4+ T lymphocytes is tightly associated with apoptosis (Terai et al., 1991; Laurent-Crawford et al., 1991, 1992b), in the course of which cellular DNA becomes fragmented at internucleosomal junctions. We have recently demonstrated that apoptosis is triggered by cell membrane expression of the mature HIV envelope glycoproteins, the gp120/gp41 complex, and their interaction with CD4 receptor molecules (Laurent-Crawford et al., 1992b, 1993). In the case of the HIV Lai isolate, syncytium formation and apoptosis induction were closely associated as both events require functional envelope glycoproteins and CD4 receptors. Despite this, we have been able to dissociate syncytium formation from apoptosis. For example, MAb OKT4 against the CD4 molecules inhibited apoptosis without affecting syncytium formation, whereas another antibody specific for the transmembrane envelope glycoprotein gp41 blocked syncytium formation without affecting the induction of apoptosis (Laurent-Crawford et al., 1993). Taken together, these observations indicate that apoptosis can occur in HIV-infected cells irrespective of syncytium formation. In accord with this, infection of CEM cells with both syncytium-inducing (HIV-2 ROD) or non-syncytium-inducing (HIV-2 EHO) cytopathic HIV-2 strains resulted in apoptosis (Rey et al., 1989a, b, unpublished results).

Apoptosis could be used as an early signal for the prediction of the c.p.e. during the HIV infection (Table 2). For example, in the synchronous type of HIV infection, apoptosis is observed at day 2 p.i., at a time of maximal production of HIV, i.e. as measured by the maximal level of viral protein synthesis. At day 2 p.i., no cell lysis occurs which is shown by the trypan blue exclusion assay. Eventually apoptotic cells disintegrate owing to cell lysis by day 4 p.i. (Table 2). Addition of AZT at 8 h p.i. blocks accumulation of the unintegrated HIV DNA due to re-infection of cells (Fig. 6) but does not affect the course of infection nor the induction of apoptosis (Table 2). On the other hand, addition of MAb specific for the V3 loop at 8 h p.i. completely blocks syncytium formation and induction of apoptosis. Cells in this latter culture will eventually die owing to cell lysis by day 5 p.i. (Table 2). Several reasons may account for lysis of single cells in the culture with the anti-V3 MAb. For example, the continual expression of high levels of viral

Table 2. Single cell killing by lysis in the presence of anti-V3 MAbs*

<table>
<thead>
<tr>
<th>Synchronous infection</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Apoptosis Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>1255</td>
<td>766</td>
<td>+ +</td>
<td>+</td>
<td>+ +</td>
</tr>
<tr>
<td>MAb 110/4 at 8 h</td>
<td>1306</td>
<td>821</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>AZT at 8 h</td>
<td>1488</td>
<td>1173</td>
<td>+ +</td>
<td>+</td>
<td>+ +</td>
</tr>
</tbody>
</table>

* CEM cells were infected with one synchronous infectious dose of HIV-1 (as in the legend of Fig. 2 to 4) and analysed for the production of virus on days 2 and 3 p.i., apoptosis on day 2 p.i. and cell lysis on days 3 and 4 p.i. Virus production was measured by the concentration of p25 in the culture supernatant. Apoptosis was monitored by the presence of histones as in the legend of Fig. 6. Cell lysis was monitored by the trypan blue exclusion assay. MAb 110/4 against the V3 loop (10 μg/ml) and AZT (5 μM) were added 8 h p.i.
RNA and protein synthesis (Somasundaran & Robinson, 1988) combined with the dramatic inhibition of cellular protein synthesis (Agy et al., 1990) both occurring during the late stages of HIV infection, may lead to severe shut-off of cellular metabolism resulting in cell lysis. Thus, the HIV-mediated c.p.e. in cell cultures, whether associated or not with apoptosis, will eventually terminate in cell lysis.

Discussion

Analogous to the c.p.e. of some retroviruses (Keshet & Temin, 1979; Weller et al., 1980; Mullins et al., 1986; Haase, 1986; Mergia & Luciw, 1991), the c.p.e. of HIV has been correlated with the accumulation of unintegrated viral DNA late in the course of virus infection (Shaw et al., 1984; Muesing et al., 1985; Pauza & Singh, 1990). The results presented here, however, rule out any correlation between the accumulation of unintegrated viral DNA and the c.p.e. of HIV. In a synchronous infection of CEM cultures, addition of AZT 6 h p.i. blocked the accumulation of the unintegrated DNA without any apparent effect on the c.p.e. or on the amount of virus produced (Fig. 3, 4 and 5). The accumulation of unintegrated HIV DNA is probably due to superinfection of cells as reported previously (Pauza & Singh, 1990; Robinson & Zinkus, 1990). In agreement with this, addition of MAbs against gp120 (which neutralize HIV infection) 6 h p.i. blocked accumulation of unintegrated HIV DNA without affecting the kinetics nor the amount of HIV particles produced (Fig. 2 and 3). These MAbs therefore blocked accumulation of unintegrated HIV DNA by inhibiting superinfection of previously infected cells.

The lack of correlation between the c.p.e. and accumulation of unintegrated HIV DNA was clearly demonstrated using this synchronous infection in which almost all cells became infected by the input virus. Under these experimental infection conditions, the c.p.e. was at its maximum at 48 h p.i., during which time more than 90% of cells were found to be producers of HIV proteins. Addition of AZT at 6 h p.i. did not modify the production of virus or the development of the c.p.e. In these experiments, the c.p.e. of HIV was monitored by the ballooning of cells and syncytium formation, and also by the presence of histones associated with low M, DNA that accumulate in the nuclei of infected cells as a consequence of apoptosis (Laurent-Crawford et al., 1991). Apoptosis, which should be differentiated from uncontrolled cell death or cell lysis, is a physiological event triggering cell death by the activation of a Ca++-dependent nuclear DNase that cleaves the chromatin at nucleosomal junctions (Duvall & Wylie, 1986). This characteristic fragmentation of the chromatin occurs in metabolically active cells and can be used as a convenient marker for cells that are condemned to die. Consequently, as apoptosis is assayed in the nuclei of intact cells, it provides an early signal of events that subsequently will lead to inhibition of cellular functions.

In a synchronous infection, the various stages of HIV replication could be investigated independently of each other. It is important to note that most previously published investigations have been based on HIV infections which last at least 5 to 6 days, i.e. under multiple cycles of infection as shown in Fig. 1. During multiple cycles of infection, only a small proportion of cells become infected by the input virus. For this reason, the course of infection lasts several days to allow time for amplification of the input virus and multiple rounds of infection. Experimental HIV infections of the multiple-cycle type are useful for comparing viral stocks but are not quite adequate in studies dealing with specific phases of HIV infection, such as accumulation of unintegrated viral DNA and the c.p.e. including cell killing. For example, a recent study which dissociated the accumulation of unintegrated viral DNA and cell killing during HIV infection was based on an infection lasting more than 5 days (Bergeron & Sodroski, 1992). Interestingly, in this latter work the authors mention that addition of AZT early during infection (up to 3 days p.i.) results in a significant reduction in viral protein expression, thus indicating that they are dealing with an infection of the multiple-cycle type. One of the major drawbacks of a multiple-cycle infection developing gradually in the cell culture is that a real event triggered by HIV infection in a small proportion of cells will not be detectable because of dilution in the larger proportion of cells that are not yet infected. Late in a multiple-cycle infection, due to a severe down-regulation of CD4 molecules, the typical c.p.e. of HIV does not occur and instead, cells start to die by lysis. This is most probably due to severe inhibition of cellular metabolism owing to high levels of viral RNA and protein synthesis (Somasundaran & Robinson, 1988) and also to a dramatic shut-off of cellular protein synthesis (Agy et al., 1990).

Unintegrated HIV DNA becomes accumulated during an acute infection as a result of superinfection of cells (Robinson & Zinkus, 1990; Pauza et al., 1990). Unintegrated HIV DNA is also detectable in peripheral blood mononuclear cells of AIDS patients not undergoing therapy. However, after a few months of anti-retroviral therapy the levels of such unintegrated DNA are significantly reduced (Dickover et al., 1992). In cell cultures, the unintegrated HIV DNA species are in three major forms: a linear copy of the viral genome with two LTRs and two circular DNA species with one or two LTRs (Kim et al., 1989; Charneau & Clavel, 1991). The precise role of the circular DNA species in the virus
replication cycle is not clear. On the other hand, it has been suggested that the linear form of DNA is the immediate precursor for integration into the host chromosome (Grandgenett & Mumm, 1990; Charneau & Clavel, 1991). Accumulation of unintegrated HIV DNA does not occur in chronically infected T cells since because of down-regulation of CD4 molecules they cannot be superinfected (Stevenson et al., 1988; Laurent-Crawford et al., 1991). Interestingly, accumulation of unusual, high M₉ extrachromosomal forms of HIV DNA has been observed in persistently HIV-infected monocytic cells (Pauza & Galindo, 1989; Pauza & Singh, 1990). Here we have demonstrated that accumulation of unintegrated HIV DNA is not correlated with the c.p.e. of HIV. Such a correlation has already been questioned in the visna virus model, with the suggestion that the envelope glycoprotein, capable of fusing from within and from outside, is a more likely candidate (Haase, 1986). This hypothesis is in agreement with data obtained in the HIV model in which the expression of viral envelope glycoproteins has been closely correlated with the c.p.e. (Sodroski et al., 1986; Lifson et al., 1986; Laurent-Crawford et al., 1992b, 1993).

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