

Reinfection Results in Accumulation of Unintegrated Viral DNA in Cytopathic and Persistent Human Immunodeficiency Virus Type 1 Infection of CEM Cells

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Summary

High levels of unintegrated viral DNA accumulate during human immunodeficiency virus type 1 (HIV-1) infection of CEM T cells. Reinfection of already infected cells is required to attain these levels and reinfection also promotes the development of HIV-induced cytopathology. Rates of virus production, however, are independent of the accumulation of unintegrated viral DNA. Neutralizing antibody added soon after infection reduced viral DNA levels without appreciably affecting the production of cell-free viral p24 antigen or reverse transcriptase activity. Only 50 pM AZT were required to reduce the accumulation of unintegrated viral DNA by 50% in contrast to the 25 nM required to inhibit virus production by 50%. Cytopathology, as measured by number of syncytia in infected cell cultures, was correlated with highly elevated levels of unintegrated viral DNA. The minimal levels of unintegrated viral DNA present constitutively in the persistently infected HCEM cell line were consonant with the absence of cytopathic effects in these cells. These data demonstrate that inhibiting the reinfection of already infected cells modulates cytopathic HIV-1 infection to a form that is persistent and noncytopathic.

Depletion of CD4 T lymphocytes is an important feature of the natural history of HIV-1 infection (1, 2). Reduced numbers in this cell population predict disease progression (1-3) and are likely to contribute to immunodeficiency (4). HIV-1 infection in vitro is cytopathic for T lymphocytes (5-7) and T lymphoid cell lines (8, 9). This cytopathology occurs by the formation of syncytia when infected and uninfected CD4⁺ cells are admixed (8, 9) and direct cell killing by a mechanism independent of cell fusion (10). Based on the cytopathic effects of HIV-1 infection of T cells in vitro, this mechanism was proposed to be a crucial feature of viral pathogenesis in AIDS (6, 5, 11, 12). In this study, we have examined the role of reinfection and viral DNA accumulation in cytopathic and persistent HIV-1 infection of the CEM cell line.

Host-pathogen interactions leading to cytopathology have been characterized extensively in avian retrovirus models. The accumulation of high levels of unintegrated viral DNA is a recognized feature of cytopathic infection by these retroviruses (13, 14) and was also shown to be an important marker of pathogenesis in the case of feline leukemia virus infection of cats (15) and equine infectious anemia virus infection of horses (16).

Unintegrated viral DNA is generated by reverse transcription of infecting viral RNA. A linear viral DNA molecule

flanked at both ends by long terminal repeat (LTR)¹ sequences (17) is generally found to be the most abundant form of unintegrated viral DNA. Intramolecular recombination between the LTR sequences then generates a circular molecule with one LTR that is of intermediate abundance. A circular DNA form with two LTR seems to arise via direct ligation of the ends of a linear molecule and is the least abundant species. Importantly, these DNA species are products of the reverse transcriptase reaction pathway and have no intrinsic capacity for replication (for a review see reference 18).

The correlation between accumulation of viral DNA and cytopathology helped to confirm that the cytopathic avian retroviruses infect, replicate, and reinfect target cells more rapidly than these cells can establish interference immunity (14). The small fraction of cells that survive the initial infection maintain reduced levels of unintegrated DNA and produce virus constitutively without evident cytopathic effects (13). These characteristics of the dynamic interaction of oncornaviruses with their host cells provided a model for investigating mechanisms of the cytopathic HIV-1 infection of T cells. Our studies confirm that reinfection of already infected cells promotes HIV-induced cytopathology. These results may

¹ Abbreviations used in this paper: LTR, long terminal repeat; moi, multiplicity of infection; TCID₅₀, tissue culture infectious dose.

help to establish parameters that will be useful for assessing cytopathic effects of HIV-1 in infected individuals and for determining the contribution of this mechanism to T lymphocyte depletion.

Materials and Methods

Cell Lines and Virus Strains. CEM cells were maintained at densities between 10^5 and 10^6 cells/ml in RPMI 1640 medium with 10% heat-inactivated FCS supplemented with penicillin/streptomycin/glutamine. The cells were passaged on the day before infection. The HCEM cell line (19) is persistently infected with the LAV-1_{bu} strain of HIV-1 (20) and produces constitutively 5×10^5 tissue culture infectious doses per ml of HIV-1, as assayed by terminal dilution in MT-2 cells (21) or in CEM cells. Cell-free supernatants of HCEM were used as inocula in all experiments.

Infection and Drug Treatments. To achieve a multiplicity of infection (moi) of 1 tissue culture infectious dose (TCID₅₀)/cell, CEM cells at 5×10^5 /ml were admixed with an equal volume of cell-free HCEM supernatant containing 5×10^5 TCID₅₀/ml. Two volumes of medium were added to this mixture, and the cells were incubated for 3–5 d.

Cells were pretreated with 3'-azido-3'-deoxythymidine (AZT, zidovudine; Aldrich Chemical Co., Inc., Milwaukee, WI) for 1 h before infection. For experiments with neutralizing antibody, ascites fluid containing the 110.4 murine mAb (22; provided by Genetic Systems, Inc., Seattle, Washington) was added directly to culture medium; the volume of ascites fluid represented 10^{-4} of the total culture volume. A dilution of 10^{-6} inhibited plaque formation (23) by 50%, and a dilution of 10^{-4} inhibited plaque formation and infectivity of CEM cells by >95% (not shown). This mAb does not block the interaction of CD4 with the gp120 envelope glycoprotein of HIV-1 (24).

Analysis of Virus and Viral DNA Accumulation. The reverse transcriptase activity of cell-free supernatants indicated the rates of virus production in infected cell cultures (5). Viral p24 antigen was quantitated by ELISA (Abbott Laboratories, N. Chicago, IL). Viral DNA

was prepared by the method of Hirt (25) and analyzed by restriction enzyme digestion and DNA blotting (19). All of the hybridization experiments were probed with radiolabeled ARV-2 cDNA (26) that contained the entire viral sequence. Size markers were included in each gel to confirm the assignments made for individual DNA species.

Results

Kinetics of Unintegrated Viral DNA Accumulation in T Cells. The presence of unintegrated, circular DNA molecules containing one or two LTR sequences and an unintegrated linear DNA form with two LTR had been demonstrated previously in CEM cells acutely or persistently infected by HIV-1. The identity of each form was confirmed by restriction mapping and blot hybridization (19). The LAV-1 genome contains a single BamHI restriction endonuclease site at position 8068 (27); thus, BamHI digestion of Hirt DNA provides a convenient method for displaying the three discrete forms of viral DNA. Subsequent to BamHI digestion, the circular DNA containing two LTR is present as a 9.8 kilobase pair (kb) fragment. The circular DNA containing 1 LTR migrates as a 9.1 kb band. The linear DNA releases two fragments upon BamHI digestion; the higher molecular weight fragment is 8.6 kb and the smaller fragment representing the 3' end of the viral genome is present as a 1.25 kb piece.

Cultures infected with 1 TCID₅₀/cell of HIV-1 were examined daily for the presence of unintegrated viral DNA (Fig. 1). Maximum viral DNA levels were attained within 4 d after infection. Fig. 1 is a short exposure of this blot hybridization in order not to darken completely the lanes representing days 3 and 4 after infection. The appearance of unintegrated viral DNA on day 1 was clearly visible in longer exposures (not shown) and is apparent in Fig. 5. The decrease in hybridiza-

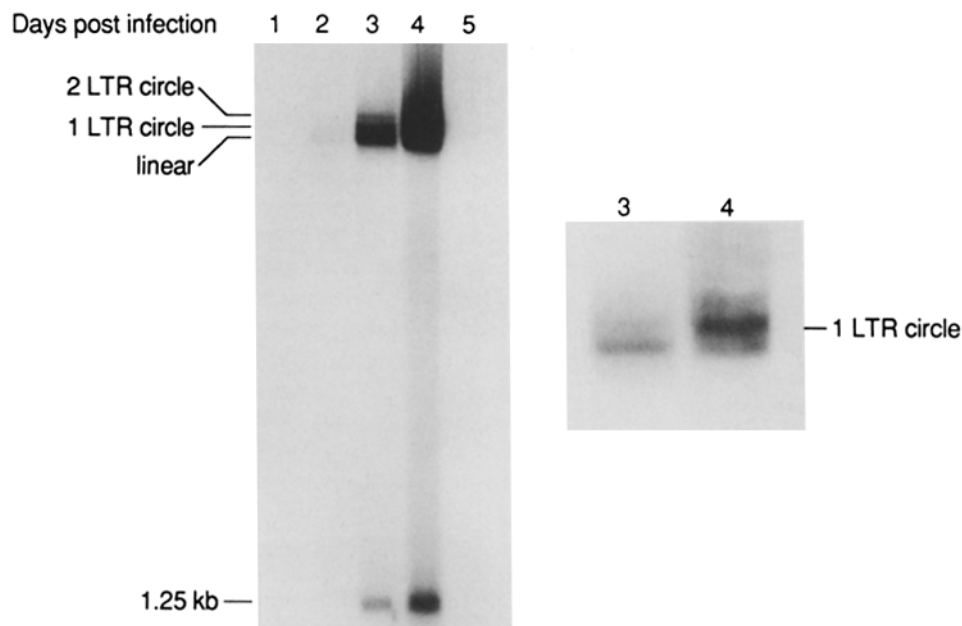


Figure 1. Accumulation of viral DNA during acute infection of CEM cells by HIV-1. Cells were infected at a multiplicity of 1 TCID₅₀ per cell. Samples were removed at daily intervals, and unintegrated viral DNA was purified by Hirt extraction (25). The DNA samples were digested with BamHI restriction endonuclease (27). The products of BamHI digestion are indicated at the left; the 1.25 kb fragment is one end of the linear molecule released by BamHI cleavage. The panel on the left shows the kinetics of viral DNA accumulation throughout 5 d of infection. The inset is an enlarged view of the samples 3 and 4 d after infection to emphasize the relative increases in 1 LTR circles.

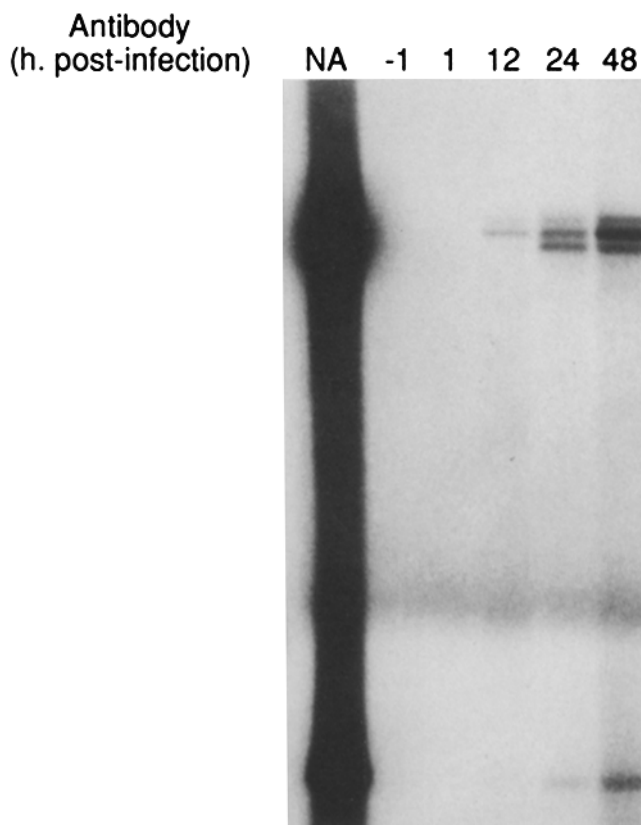


Figure 2. Neutralizing antibody prevents accumulation of viral DNA during acute infection. Neutralizing mAb 110.4 (24) was added at various intervals before and after infection and cells were cultured in medium containing the antibody. 3 d after infection, samples were collected and DNA was extracted, digested, and analyzed by blot hybridization as for Fig. 1. The values at the top of the figure indicate the times of antibody addition in hours after infection. The value of -1 indicates treatment with neutralizing antibody 1 h before virus infection. The designation NA represents the degree of viral DNA accumulation in the absence of neutralizing antibody.

tion intensity at day 5 after infection is due mainly to the loss of viable cells in the culture.

Effects of Neutralizing Antibody on Viral DNA Accumulation and Cytopathology. In previous studies of cytopathic avian

retroviruses, neutralizing antibody added after infection blocked reinfection of already infected cells and prevented viral DNA accumulation and cytopathology (13, 14). To explore the hypothesis that reinfection is essential for viral DNA accumulation and cytopathology in T cell cultures, neutralizing mAb was added at various intervals before and after infection of CEM cells with HIV-1. We then examined viral DNA accumulation, virus production, and the extent of cytopathic effects in these cultures. The mAb 110.4 was used in these experiments. This antibody recognizes an epitope located between amino acids 303 and 323 of the viral envelope protein and inhibits virus infection without blocking binding of gp120 to the CD4 receptor (24). The 110.4 mAb is type-specific in that it neutralizes only the LAV-1_{bru} strain of HIV-1 (22).

The levels of unintegrated viral DNA were examined by blot hybridization at various intervals after adding antibody to infected cell cultures (Fig. 2). The input moi used in these experiments (1 TCID/cell) yielded at least 30% of the cells as virus antigen-positive within 24 h (not shown). Addition of mAb 110.4 before infection eliminated all detectable viral DNA accumulation, thereby confirming the neutralizing activity of this antibody. Antibody added within 48 h after infection reduced significantly the viral DNA levels. The magnitude of this effect was correlated with the time of antibody addition; antibody added 1-12 h after infection inhibited viral DNA accumulation to a greater extent than antibody added 24-48 h after infection. Neutralizing mAb added 12 or more hours after infection had no significant effect on cell-free reverse transcriptase activity or p24 antigen accumulation (Table 1).

Cytopathology in HIV-1-infected CEM cell cultures was measured by the appearance of multinucleated, giant cell syncytia (9, 28). The frequency of syncytia was reduced significantly when infected cells were exposed to the 110.4 neutralizing mAb either before or after infection (Table 2). In general, the decrease in syncytium frequency was correlated with reduced levels of unintegrated viral DNA; however, some syncytia were noted in the cultures that received antibody at 1 h after infection, even though viral DNA was not detected by blot hybridization of these samples.

Effects of AZT on Viral DNA Accumulation, Virus Production, and Cytopathology. The impact of reinfection and viral DNA accumulation was also examined by analyzing the effects

Table 1. Virus and Viral Antigen Production in Cells Treated with Neutralizing mAb

Antibody addition (h, after infection)	NA*	-1*	1	12	24	48
Reverse transcriptase [†]	19 ± 3	0.9 ± 0.3	12 ± 2	16 ± 2	18 ± 4	23 ± 5
p24 Antigen [‡]	102	<1	45	48	51	116

* NA indicates that antibody was not added to this sample and -1 represents a 1-h pretreatment with antibody before virus infection.

† Virus production assessed by reverse transcriptase assay of cell-free culture supernatants collected 3 d after infection. Units are 10³ cpm of [³²P]TTP incorporated in a 2-h assay (49) and are the averages of triplicate determinations from each of three independent infection experiments.

‡ The data are represented as nanograms of p24 per milliliter of culture supernatant. The standard error in p24 determinations did not exceed 15% of the mean values.

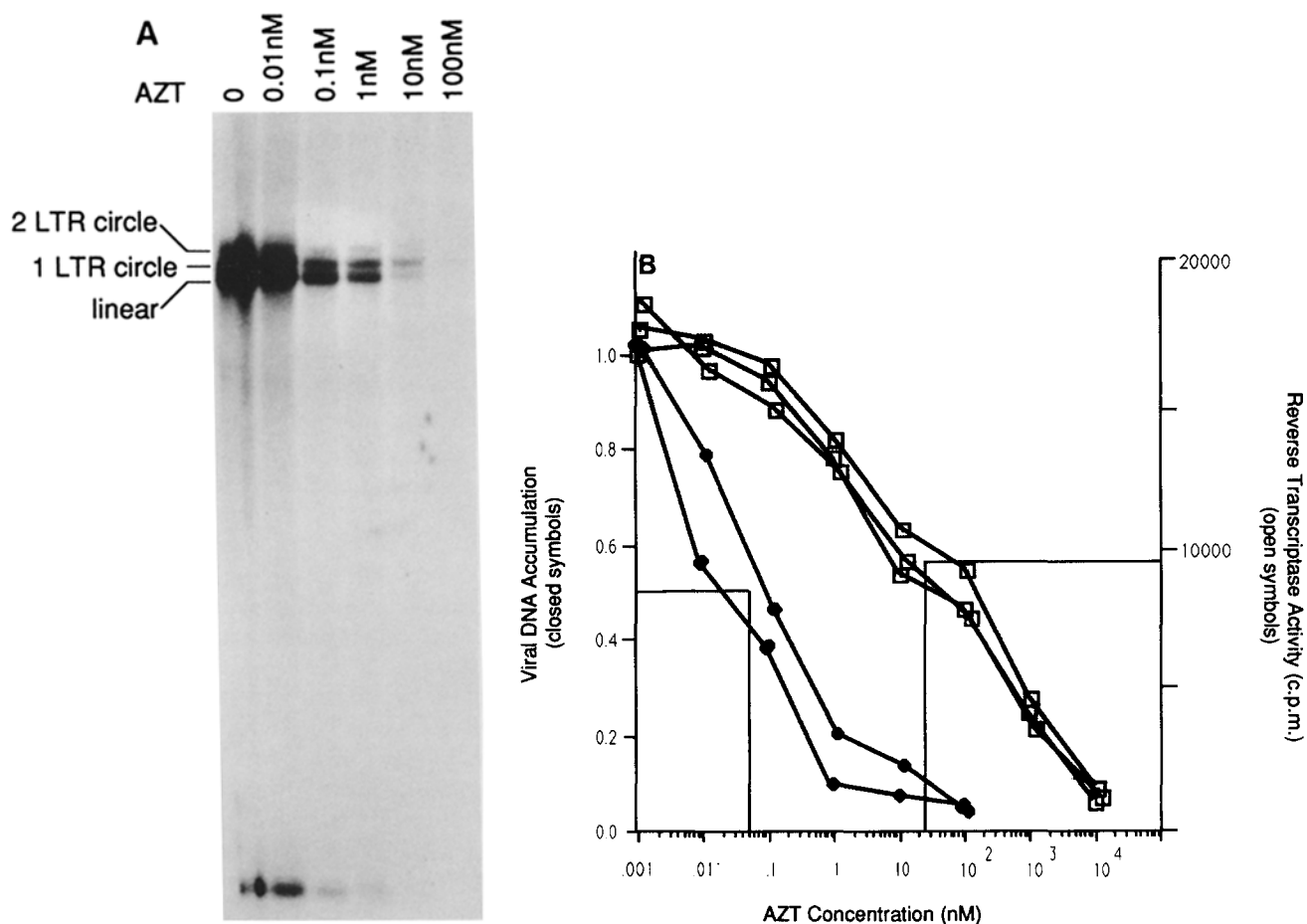


Figure 3. Inhibition of HIV-1 infection by 3'-azido-3'-deoxythymidine. CEM cells were treated with AZT for 1 h and then infected with HIV-1 at a multiplicity of 1 TCID₅₀/cell. Viral DNA and cell-free supernatants were obtained from cultures 3 d after infection. (A) Blot hybridization of unincorporated viral DNA accumulated in the presence of AZT; the DNA samples were digested with BamHI restriction endonuclease. The positions of circular and linear DNA species are indicated at the left and the AZT concentration (nM) is shown at the top. (B) Quantitation of virus production and unincorporated DNA accumulation 3 d after infection at varying concentrations of AZT. Viral DNA levels (filled symbols) were quantitated by removing and counting radioactive bands from the hybridization membrane; areas of the membrane containing circular and linear viral DNA were excised. The data were normalized to a value of 1 for the no drug treatment samples. Cell-free reverse transcriptase activity was assayed (49) and is indicated by the open symbols. The results for reverse transcriptase assays are the averages of triplicate determinations. Three independent infections were assayed for reverse transcriptase activity and two independent infection experiments were quantitated for viral DNA accumulation.

of AZT on HIV-1 infection of CEM cells. Virus production measured by cell-free reverse transcriptase activity was examined 3 d after infection as a function of the AZT concentration added before infection (Fig. 3). Virus production was inhibited 50% at 25 ± 16 nM AZT; 5 μM AZT was required to inhibit virus production by 90% (Fig. 3 A). The value of 25 nM AZT for 50% inhibition of virus production is in accord with inhibitory concentrations measured by p24 production (29), plaque formation (23), or cell-free reverse transcriptase activity (30).

Similar dose-response experiments were then performed to evaluate AZT effects on viral DNA accumulation 3 d after infection. The addition of 0.01 nM to 100 nM AZT before infection inhibited HIV-1 DNA accumulation (Fig. 3 B). By counting radioactivity in bands of DNA excised from hybridization membranes, these experiments yielded quantita-

tive information showing that viral DNA accumulation was inhibited by 50% at 60 pM AZT and by 90% at 10 nM AZT (Fig. 3 A). We also tested AZT doses as high as 100 μM and as low as 0.1 pM (not shown); the concentration range shown here was selected to emphasize the linear portion of the dose-response curve for inhibition of viral DNA accumulation by AZT. Adding AZT before infection also inhibited cytopathology in infected cell cultures. Significant reduction in syncytium frequency at 3 d after infection was seen with as little as 10 nM AZT in the culture medium (Table 3).

Effects of Neutralizing Antibody or AZT on Viral DNA Accumulation in a Persistently Infected T Cell Line. In addition to initiating acute, cytopathic infection of CEM cells, HIV-1 can also establish a persistent infection in these cells (19). The cloned cell line HCEM, for example, contains a single integrated provirus (not shown) and is fully capable of forming

Table 2. Neutralizing mAb Added after Infection Inhibits Syncytium Formation in Infected CEM Cells

Antibody addition (h, after infection)	NA	-1	1	12	24	48
Syncytia/field*	78	0	12	13	17	20
Range of values	71-125	0	10-21	8-21	11-26	19-45

* Syncytia 3 d after infection were counted within a single field observed using lower power objectives (total magnification, $\times 200$). Syncytia were defined because they were multinucleated or they were larger than three cell diameters; at least five fields were counted for each sample. Values given in the first row represent the numerical averages of syncytium frequency data obtained from observing four independent infection experiments. The values from independent data sets were averaged, after discarding the single value farthest from the mean.

syncytia in the presence of uninfected CD4⁺ cells, although this cell line resists homotypic fusion. During logarithmic growth, HCEM cultures produce $\sim 5 \times 10^5$ TCID₅₀/ml of infectious HIV-1 in the culture medium.

The observation of unintegrated viral DNA in HCEM cells (19) prompted investigation of the mechanisms responsible for maintaining these DNA species. The effects of AZT or neutralizing mAb on unintegrated viral DNA were examined. HCEM cells were washed and recultured in fresh medium to reduce as much as possible the input titer of soluble virus. Unintegrated viral DNA decreased within 1 d after AZT treatment of these washed cells (Fig. 4). There was very little effect of AZT on viral DNA levels at 3 d after treatment. AZT

Table 3. AZT Inhibits Syncytium Formation in Infected CEM Cells

	AZT concentration (nM)					
	0	0.1	1	10	100	1,000
Syncytia/field (average)*	86	91	83	32	23	11
Range of values	73-141	77-108	78-115	21-52	13-31	4-23

* Syncytia at 3 d after infection were counted within a single field observed using low power objectives (total magnification, $\times 200$). Syncytia were defined because they were multinucleated or they were larger than three cell diameters; at least five fields were counted for each sample. Values shown in the first row represent the numerical averages of syncytium frequency data obtained from four independent infection experiments. The values from independent data sets were averaged, after discarding the single value farthest from the mean.

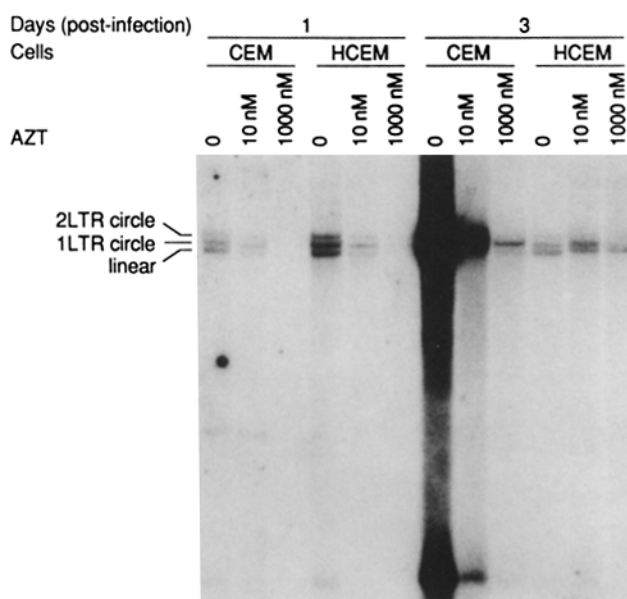


Figure 4. Time course of inhibition of viral DNA accumulation in acutely and persistently infected CEM cells by AZT. AZT was added to cultures 1 h before the addition of virus (acute infection lanes are labeled CEM). Drug was added to the medium of persistently infected HCEM after washing these cells to remove exogenous virus. The concentrations of AZT are indicated. Samples were collected 1 or 3 d later and viral DNA was extracted, digested, fractionated by electrophoresis, and analyzed by blot hybridization as in Fig. 1. The positions of the three species of viral DNA are indicated at the left.

treatment also had no effect on virus production by HCEM cells (not shown), a result consistent with previous observations by others (31, 32). Apparently, constitutive virus production combined with what may have been diminishing AZT concentrations in the medium, permitted accumulation of viral DNA to near normal levels within 3 d after drug treatment.

Neutralizing mAb 110.4 was also utilized to block reinfection of HCEM cells. Evaluation of the levels of unintegrated viral DNA 3 d after treatment supported the view that reinfection leads to viral DNA accumulation in HCEM cells (Fig. 5). Viral DNA levels in HCEM were significantly lower than the levels seen in cells acutely infected with HIV-1 for 3 d (Figs. 4 and 5).

Discussion

The accumulation of unintegrated viral DNA is a characteristic of the acute, cytopathic HIV-1 infection of T cells (19, 33, 34). In this study, similar accumulation of unintegrated HIV-1 DNA was shown to occur over 3 d after infection by a mechanism requiring reinfection of already infected cells. This accumulation of viral DNA correlated with the extent of cytopathology in the cultures. HIV-1 is thus similar to the cytopathic avian oncornaviruses which infect, replicate, and reinfect target cells more rapidly than these cells establish interference immunity (13, 14). Addition of neutralizing

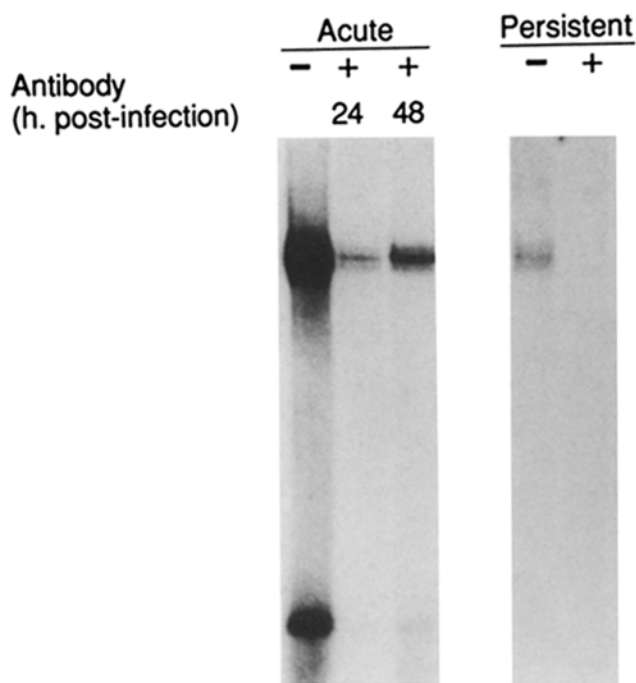


Figure 5. Neutralizing antibody prevents accumulation of viral DNA in persistently infected HCEM cells. Infection and analysis of DNA were as described for Fig. 4. The acute and persistent infection lanes are indicated at the top along with (-) to indicate no antibody in the culture or (+) to show the presence of antibody. For the acute infection, the hours after infection for antibody addition are indicated; the cells were harvested at 3 d after infection. The persistently infected cells were washed and resuspended in fresh medium containing antibody, then cultured for 3 d and collected for analysis of DNA.

mAb or AZT to already infected cells reduced the levels of unintegrated viral DNA without affecting the rates of virus production. Therefore, unintegrated DNA does not contribute significantly to the rates of virus production and does not promote cytopathic effects by spreading the infection within the culture. It is possible that transcription of unintegrated DNA might generate viral gene products that promote syncytium formation or are directly toxic to cells. It is also possible that unintegrated DNA itself might induce a lethal cellular process. However, it remains a formal possibility that viral DNA accumulation is only associated with developing cytopathology and is not a direct cause of cell death.

This characterization of cytopathic HIV-1 infection may explain some of the recognized features of AZT activity. We and others (31, 32) have reported that AZT treatment does not inhibit virus production in persistently infected T cell lines. Initially this result seemed to conflict with the observation that AZT inhibited viral DNA accumulation in these cells. The analysis of virus production in cells treated after infection with neutralizing mAb indicated that virus production rates were independent of unintegrated viral DNA accumulation. Accordingly, AZT treatment failed to affect HIV-1 production by persistently infected HCEM cells.

Smith et al. reported that T cell cultures infected and maintained in the presence of 25 μ M AZT showed rates of HIV-1

replication 20 d after infection identical to infected cultures not treated with AZT (35). Apparently, the initial infection was efficiently though incompletely inhibited by AZT. Continued presence of the drug reduced reinfection of the small fraction of already infected cells and prevented the development of cytopathology. The infected cells were thus able to survive and to produce virus that slowly spread throughout the culture. Within 20 d after infection this slow process apparently had proceeded to the point where virus production in the treated cultures equalled the rates of virus production in cultures infected in the absence of AZT.

In addition to its demonstrated value as an antiviral agent for the treatment of HIV-1 infection (36, 37), AZT also has the potential to modulate cytopathic HIV-1 infection of T cells to a form that is persistent and noncytopathic. This feature of drug action is due to inhibiting reinfection of already infected cells and requires lower concentrations of drug than are necessary to inhibit primary infection. This difference is probably attributable to the fact that accumulation of unintegrated viral DNA and cytopathology result from multiple, cumulative reinfections only a proportion of which must be inhibited to modulate the infection. In the presence of AZT, infected cells survive and continue to produce virus because they are not destroyed by viral cytopathic effects. However, removal of AZT from cultures infected in the presence of the drug resulted in rapid development of cytopathology (not shown). Therefore, AZT treatment modulates the effects of HIV-1 infection and generates a type of persistent infection that is transient and depends on continued presence of the drug.

The explanation for viral persistence in HCEM cells also involves limitations on the rate of reinfection, even though this phenomenon is independent of exogenous antiviral agents. The steady-state levels of viral DNA in HCEM are reduced considerably compared with the levels found in acutely infected cells at day 3. This finding is similar to the situation for cells chronically infected by avian retroviruses (13, 14) and reflects an important cellular adaptation to infection that facilitates establishment of virus persistence. HIV-1-infected cells are known to have decreased numbers of surface CD4 receptors (38-41) and similar observations have been made for HCEM cells (not shown). Reduced receptor levels appear to be a key alteration in this cell line that decreases reinfection rates. Therefore, antiviral agents or decreased receptor levels can limit reinfection of already infected cells and promote the establishment and maintenance of persistent HIV-1 infection of T cells. Importantly, these mechanisms operate on virulent HIV-1 strains and do not require selection of mutationally altered viruses (42) in order to establish a persistent infection.

This model for the role of reinfection and unintegrated viral DNA in HIV-1 cytopathology may not apply to the situation of monocytes and macrophages. The unintegrated viral DNA present in persistently infected monocytic cells is structurally distinct from the viral DNA forms discussed here and appears to replicate autonomously (19).

It is unclear how the *in vitro* observations resulting from these studies apply to T cell destruction *in vivo*. The interplay of infectious, circulating free virus (43, 44), neutralizing antibody, and cellular heterogeneity in different tissues make

the in vivo relationships difficult to characterize. Nevertheless, initial efforts to document the accumulation of unintegrated retroviral DNA and to correlate this with pathology (15, 16, 45) suggest that the processes described in our studies may apply to the situation in vivo. Moreover, recent observa-

tions of beneficial effects of lower AZT doses (46-48) may relate to our observations of the effects of AZT on cytopathology at concentrations significantly lower than those required to inhibit primary infection.

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