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Dissociation of Unintegrated Viral DNA Accumulation from Single-Cell Lysis Induced by Human Immunodeficiency Virus Type 1

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Acute cytopathic retroviral infections are accompanied by the accumulation, due to superinfection, of large amounts of unintegrated viral DNA in the cells. The cytopathic effects of human immunodeficiency virus type 1 (HIV-1) infection are specific for cells that express the CD4 viral receptor and consist of syncytium formation and single-cell lysis. Here we investigated the relationship between superinfection and single-cell lysis by HIV-1. Antiviral agents were added to C8166 or Jurkat lymphocytes after HIV-1 infection had occurred. Treatment with azidothymidine or a neutralizing anti-gp120 monoclonal antibody reduced or eliminated, respectively, the formation of unintegrated viral DNA but did not inhibit single-cell killing. Furthermore, in the infected Jurkat cells, the levels of unintegrated viral DNA peaked several days before significant single-cell lysis was observed. Essentially complete superinfection resistance was established before the occurrence of single-cell killing. These results demonstrate that single-cell lysis by HIV-1 can be dissociated from superinfection and unintegrated viral DNA accumulation. These results also indicate that single-cell killing may involve envelope glycoprotein-receptor interactions not accessible to the exterior of the cell.

AIDS is characterized by a depletion of CD4-positive lymphocytes, resulting in a defective cellular immune response (9, 11, 12, 28, 29). Human immunodeficiency virus type 1 (HIV-1), the etiologic agent of AIDS (2, 10, 38a), infects and kills CD4-positive lymphocytes in tissue culture. Recent estimates of virus burden in HIV-1-infected individuals make it likely that viral cytopathic effect contributes, at least in part, to lymphocyte depletion in vivo (3, 5, 17, 19, 45).

The tropism of HIV-1 for CD4-positive lymphocytes is due to the high-affinity binding of the viral exterior envelope glycoprotein gp120 and the virus receptor, CD4 (4, 7, 24, 34). The gp120 exterior envelope glycoprotein and the gp41 transmembrane envelope glycoprotein are derived by cleavage of a gp160 precursor (1, 42). Following receptor binding, gp120 and gp41 mediate the fusion of viral and host cell membranes to allow virus entry into the target cell (15, 27, 51). Similar processes mediated by the HIV-1 envelope glycoproteins expressed on the surface of infected cells lead to fusion of these cells with surrounding CD4-positive cells (31, 47). The resulting syncytia exhibit nuclear pyknosis and membrane fragility and eventually die (31, 47).

Single-cell lysis is the predominant form of cell killing associated with cytopathic retroviruses other than HIV-1. Lytic retrovirus infection is typically accompanied by accumulation of high levels of unintegrated viral DNA (38, 49), which results from superinfection of target cells (13, 43). The accumulation of unintegrated viral DNA correlates with cell killing and/or disease induction for avian leukosis virus (44, 59), spleen necrosis virus (21), feline leukemia virus (37), and equine infectious anemia virus (41). The formation of unintegrated viral DNA, which includes a linear form as well as single-long-terminal-repeat (LTR) and two-LTR circular forms (8, 46), is checked by the development of superinfection resistance (58). It has been reported that target cell lysis and extrachromosomal viral DNA accumulation can be inhibited by treatment of infected cells with neutralizing antibodies, suggesting the hypothesis that the unintegrated viral DNA contributes directly or indirectly to cytotoxicity (59).

Single-cell lysis, in addition to syncytium formation, contributes to the cytopathic effect associated with HIV-1 infection (27, 48). Single-cell lysis occurs later in the course of in vitro infection than does syncytium formation and is dependent upon CD4 expression (7b). The latter requirement is supported by the observation that cells expressing very low levels of CD4 may emerge following HIV-1 infection and exhibit little or no cytopathic effect despite the production of large amounts of cytopathic HIV-1 (27). These results also indicate that the levels of viral RNA or protein production or the release of virus particles is insufficient per se to account for single-cell lysis by HIV-1. This assertion is further supported by the identification of mutant HIV-1 viruses that can replicate comparably to the wild-type virus but are attenuated for induction of cytopathic effect. A decreased rate of envelope glycoprotein precursor processing was found in a replication-competent HIV-1 mutant that formed syncytia but exhibited less accumulation of unintegrated viral DNA and less single-cell lysis (52). A decrease in the ability of the HIV-1 envelope glycoproteins to fuse membranes, resulting from a change in the gp41 amino terminus, was associated with a virus that achieved replication levels comparable to those of the wild-type virus but was attenuated for both syncytium formation and single-cell lysis (27). These results suggest that envelope precursor cleavage, which activates the fusogenic capacity of the envelope glycoproteins (33), and membrane-fusing ability are essential for single-cell lysis. The precise context in which envelope glycoprotein-receptor interactions contribute to single-cell

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lysis by HIV-1 and whether membrane fusion per se contributes to lysis or merely serves as a means for the efficient accumulation of toxic levels of viral DNA are unknown. Here, we investigate these questions.

MATERIALS AND METHODS

Reagents. Soluble CD4 was obtained from American Biotechnology Inc. OKT4 and OKT4A were purchased from Ortho Diagnostics. The 110.4 antibody was provided by Genetic Systems, Inc. (Seattle, Wash.). Zidovudine (azidothymidine [AZT]) was obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wis.).

Infection and cytopathogenicity studies. All infections were done with the HXBc2 clone of HIV-1. The supernatant from Jurkat cells infected with the HXBc2 strain was used as a source of virus. For infection, C8166 or Jurkat cells were resuspended at a density of 10^6 cells per ml in supernatant from HIV-1-producing cells. HIV-1-producing cells were passaged the day before infection to ensure that fresh virion particles (7×10^4 to 8×10^4 cpm of reverse transcriptase activity per ml) were present in the supernatant. Infected cells were passaged daily to maintain cell density in the range of 1×10^6 cells per ml for Jurkat lymphocytes and 5×10^5 cells per ml for C8166 lymphocytes.

Cytopathic effect was measured by counting the number of syncytia and the percentage of trypan blue-staining single cells as described previously (27). Antiviral agents were added to the culture at the concentrations and time points (8 h postinfection for C8166 lymphocytes and 3 or 4 days postinfection for the Jurkat lymphocytes) indicated and were kept in the culture medium for the duration of the study.

Viral protein expression. To assess viral protein expression, 3×10^6 viable (trypan blue-excluding) cells were radiolabeled with 100 μCi of ^{35}S-cysteine per ml overnight and immunoprecipitated with an excess of serum from a patient with AIDS (27). Expression of viral proteins was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and autoradiography.

Cell surface viral protein expression was determined by fluorescence-activated cell sorter analysis with a monoclonal antibody against gp120 (antibody 110.4) (22) and a fluorescein isothiocyanate (FITC)-conjugated mouse anti-human immunoglobulin monoclonal antibody (Sigma). To estimate the number of infected cells in the culture, we performed an immunofluorescence assay with fixed cells. Approximately 10^5 cells in a volume of 100 μl of phosphate-buffered saline (PBS) were applied to an eight-ring slide (Belko) coated with poly-L-lysine. The slides were incubated at 37°C for 30 min to allow the lymphocytes to adhere and then washed with PBS. The cells were fixed with 2% paraformaldehyde–0.1% Triton X-100 for 10 min at room temperature. The slides were washed twice with PBS and twice with PBS–10% fetal calf serum. A concentration of 0.8 μg of 110.4 antibody per ml in a volume of 100 μl of PBS–10% fetal calf serum was added to the slide for 45 min on ice. The slides were washed twice with PBS–10% fetal calf serum, and a FITC-conjugated mouse anti-human immunoglobulin monoclonal antibody (Sigma) diluted 1:50 (10 μg/ml) was added. The slides were incubated for 45 min on ice, washed four times with PBS, and viewed under a fluorescence microscope (Nikon).

Replication complementation assay. Complementation of the single-step replication of the envelope-deficient chloramphenicol acetyltransferase (CAT)-expressing provirus HXBΔenvCAT was performed as described before (15). COS-1 cells were transfected with 5 μg each of HXBΔenv CAT and the envelope expressor plasmid pSVIIIenv. Equivalent amounts of reverse transcriptase activity representing recombinant virions in COS-1 supernatants were added to Jurkat cells. To test the effect of antiviral agents, 0.6 ml of COS-1 supernatant was incubated with soluble CD4 or 110.4 monoclonal antibody for 1 h at 37°C and added to 10^6 Jurkat cells. In the case of treatment with OKT4A antibody, the Jurkat cells were incubated with antibody for 1 h at 37°C before infection. For AZT, Jurkat cells were incubated with different concentrations of the drug for 12 h prior to infection. At 72 h following infection, an equal number of target cells were lysed, and CAT activity was measured as described before (15). The background value obtained with the negative control, pSVIIIenvΔKS, which does not express a functional HIV-1 envelope glycoprotein, was subtracted from all other values.

Inhibition of gp120 binding to CD4 by OKT4A antibody. COS-1 cells were transfected by the DEAE-dextran method with pSVIIIenv DNA, expressing envelope glycoproteins, as described before (6, 15). The cells were radiolabeled with ^{35}S-cysteine overnight, and the supernatant containing gp120 was incubated with an excess of SupT1 lymphocytes at 37°C for 90 min. The SupT1 cells were preincubated with various concentrations of OKT4A antibody for 1 h at 37°C. The cells were washed twice with PBS, lysed, and immunoprecipitated with serum from an AIDS patient. The amount of gp120 precipitated from both the bound and unbound fractions was measured by SDS-acrylamide gel electrophoresis.

Measurement of unintegrated viral DNA. Viral DNA was prepared by the method of Hirt (16) and analyzed by restriction enzyme digestion and DNA blotting (50). Approximately 2×10^7 viable cells were harvested, washed once with PBS, resuspended in 7 ml of 10 mM Tris (pH 7.4)–10 mM EDTA, and incubated at 65°C for 15 min. One milliliter of 10% SDS was added, and the lysed cells were incubated for 15 min at 65°C. Chromosomal DNA and proteins were precipitated by addition of 2.5 ml of 5 M NaCl and stored at 4°C overnight. After centrifugation at 14,000 × *g* for 30 min, the supernatant was collected, and the unintegrated viral DNA was purified by ethanol precipitation, phenol extraction, and RNase A (20 μg/ml) digestion. Half of the DNA obtained from the Hirt preparation was loaded on the gel undigested, while the remainder of the DNA was digested with *Pst*I and *Sal*I prior to gel analysis. Ethidium bromide-stained gels were analyzed to ensure that equivalent amounts of DNA were loaded in each lane. The Southern blots were probed with a 551-bp radiolabeled *Hin*dIII fragment of the HXBc2 provirus clone encompassing the LTR, leader sequences, and the 5′ portion of the *gag* gene (nucleotides 80 to 631 of the Ratner et al. [40] sequence).

Assessment of superinfection resistance. Jurkat cells were infected with HXBc2 virus as described above. At various times after infection, 2×10^6 cells were infected with a replication-competent HIV-1 expressing the CAT gene (HIV-CAT) (56). The target cells were resuspended in 2 ml of supernatant from Jurkat cells producing HIV-CAT (1.5×10^5 to 2.0×10^5 cpm of reverse transcriptase activity per ml). The cells were resuspended in fresh medium the next day, and the amount of CAT activity produced by the cells was measured 72 h after infection with the HIV-CAT virus.

RESULTS

Effects of superinfection block on cytopathic effect in C8166 cells. To examine the role of superinfection in HIV-1 cytopathic effect, C8166 lymphocytes were infected with the

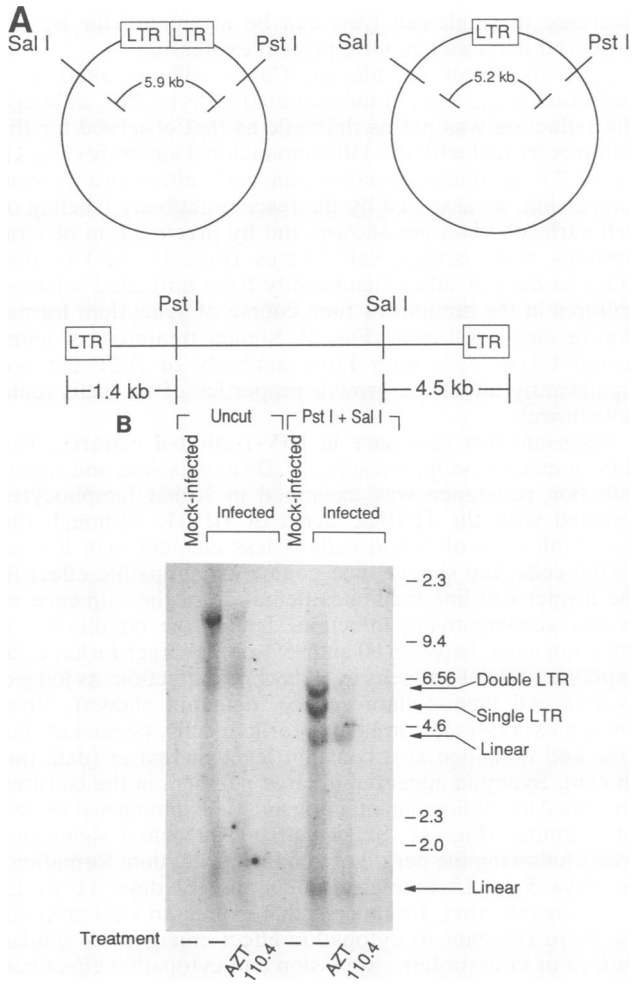

FIG. 1. Effect of AZT and 110.4 antibody on unintegrated viral DNA levels in HIV-1-infected C8166 cells. (A) Diagrams of the three forms of unintegrated viral DNA expected in HXBc2-infected cells and predicted sizes, after *Pst*I and *Sal*I digestion, associated with each form in a Southern blot. (B) Unintegrated viral DNA from Hirt supernatants observed in a Southern blot 2 days following infection of C8166 cells with the HXBc2 virus. The cells were either untreated or treated with 10 nM AZT or 20 μg of 110.4 monoclonal antibody per ml beginning at 8 h postinfection. The positions of molecular size markers (in kilobase pairs) and expected fragment sizes are indicated.

HXBc2 strain of HIV-1. Under the infection conditions used, all of the C8166 cells expressed HIV-1 antigens by 36 h postinfection, as judged by live cell immunofluorescence (data not shown). Unintegrated HIV-1 DNA, which accumulates as a result of superinfection of infected cells (43), was evident on day 2 following infection (Fig. 1). Linear viral DNA, which is the precursor for integrated provirus, and one- and two-LTR circular forms of unintegrated viral DNA (8, 46) were detected. Syncytia were observed in the infected C8166 cultures from 1 to 3 days following infection and decreased rapidly in size and number thereafter. Single-cell lysis increased until days 4 to 5 after infection, at which time all of the cells demonstrated cytopathic changes (Fig. 2). No viable cells were evident in the HIV-1-infected cultures by 7 days after infection.

To examine the requirement for superinfection for HIV-1

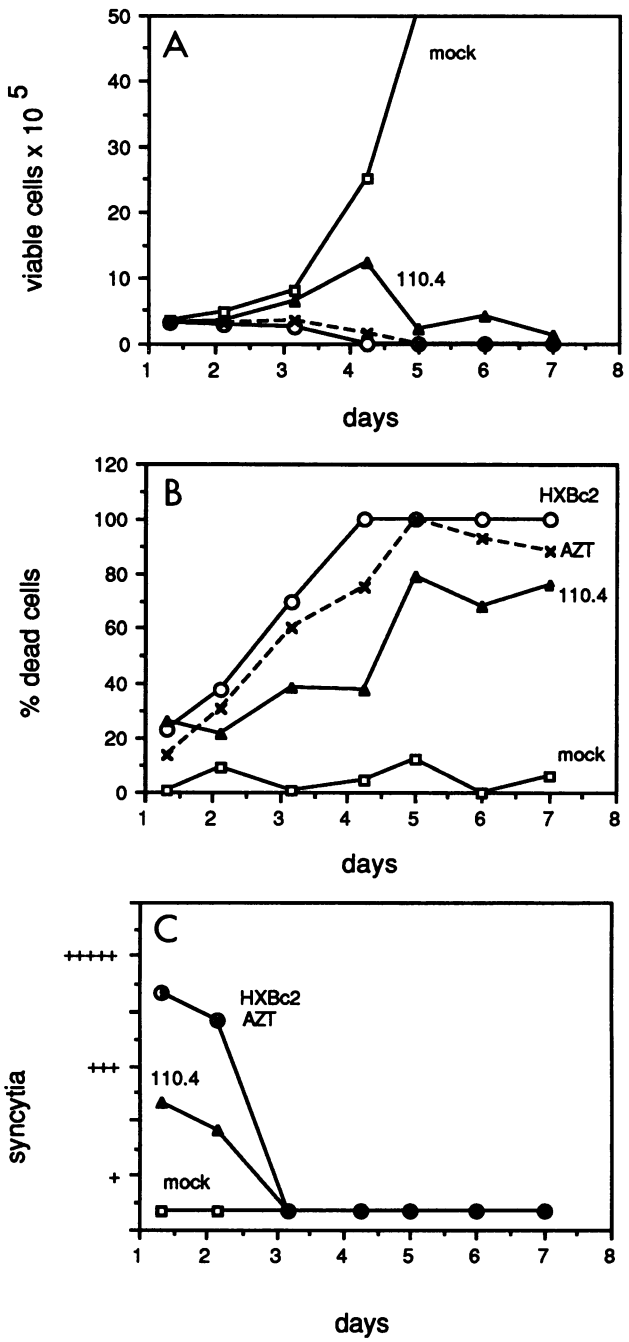

FIG. 2. Cytopathic effects of HIV-1 infection of C8166 lymphocytes. (A) Effect of HIV-1 infection on viability of the C8166 lymphocytes infected with the HXBc2 strain of HIV-1 (○) compared with that of mock-infected C8166 cells (□). Seven days after infection, the mock-infected culture had reached a total of 1.2×10^7 cells. Also shown is the effect of treatment with 10 nM AZT (×) and 20 μg of 110.4 monoclonal antibody per ml (▲) at 8 h postinfection. (B and C) Percentage of single cells that stained positive with trypan blue (B) and the number of syncytia observed (C). Syncytia were scored as follows: +, 1 to 2 syncytia per 5 high-power (100×) fields in a culture of 5×10^5 cells per ml; +++, 10 to 20 syncytia per field; +++++, 35 to 45 syncytia per field.

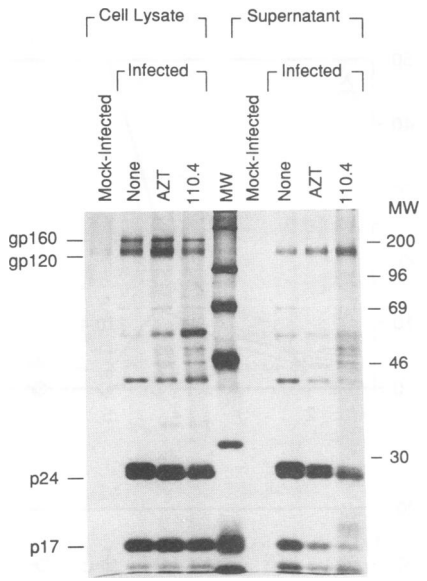

FIG. 3. Effect of AZT and 110.4 antibody on viral protein expression in HIV-1-infected C8166 cells. Infected C8166 cell cultures treated as described in the legend to Fig. 2 were analyzed at 2 days postinfection for viral protein expression. Lane MW, size markers (in kilodaltons).

protein expression and cytopathic effect, C8166 lymphocytes were infected with HIV-1 under the conditions described above. Eight hours after infection, either the 110.4 monoclonal antibody or AZT was added to the cultures to inhibit superinfection. The 110.4 monoclonal antibody binds the HIV-1 gp120 V3 loop and neutralizes virus infection by interfering with a process other than receptor binding (22, 32, 46b). AZT inhibits HIV-1 reverse transcription (34a). Treatment of the infected C8166 cultures with the 110.4 monoclonal antibody reduced the level of unintegrated HIV-1 DNA to almost undetectable levels on day 2 following infection (Fig. 1). This treatment had mild effects on the total amount of viral protein in the cultures, measured at 2 days following infection (Fig. 3). The expression of the HIV-1 envelope glycoproteins on the surface of infected C8166 cells at that time was not altered by the 110.4 antibody treatment, as determined by live cell immunofluorescence (data not shown). This result is consistent with those of other studies indicating that a significant reduction in total HIV-1 envelope glycoprotein expression is needed to reduce cell surface expression levels (56). In addition, this result indicates that the antibody treatment did not reduce the percentage of cells that express HIV-1 proteins in the culture.

The appearance of cytopathic effects in the 110.4 antibody-treated cultures was slightly delayed relative to that in untreated, infected cultures, but the antibody treatment did not prevent cell killing (Fig. 2). A reduction in the number of syncytia was observed in 110.4 monoclonal antibody-treated cultures, consistent with the reported ability of this antibody to inhibit HIV-1-mediated syncytium formation (22). The degree of single-cell lysis in the antibody-treated cultures was only marginally decreased relative to that observed in the untreated cultures. Thus, in this system, a dramatic reduction in the level of unintegrated viral DNA, which reflects an efficient inhibition of superinfection, moderately decreased viral protein expression but did not prevent viral cytopathic effect. The delay in cytopathic effect and slight decrease in single-cell lysis can be accounted for by the observed decreases in viral protein expression.

AZT treatment of infected C8166 cells resulted in a reduction in the level of unintegrated HIV-1 DNA, although the reduction was not as dramatic as that observed for the cultures treated with the 110.4 monoclonal antibody (Fig. 1). The AZT treatment did not significantly affect viral protein expression, as assessed by fluorescent-antibody labeling of cell surfaces (data not shown) and by precipitation of viral proteins from labeled cell lysates (Fig. 3). AZT-treated cultures did not differ significantly from untreated infected cultures in the amount or time course of syncytium formation or single-cell lysis (Fig. 2). Similar treatment of uninfected C8166 cells with 110.4 antibody or AZT did not significantly affect the growth properties of the cells (data not shown).

Superinfection resistance in HIV-1-infected cultures. The time course of cytopathic effect, CD4 expression, and superinfection resistance was examined in Jurkat lymphocytes infected with the HXBc2 strain of HIV-1. Although the initial infection of Jurkat cells is less efficient than that of C8166 cells, the slower time course of cytopathic effect in the former cell line facilitates definition of the sequence of events accompanying infection. Under the conditions of infection used, between 80 and 95% of the target Jurkat cells expressed HIV-1 antigens by 3 days postinfection, as judged by live cell immunofluorescence (data not shown). Viral protein expression, normalized for live cells, peaked at this time and remained at a constant level thereafter (data not shown). Syncytia appeared in large numbers in the cultures on days 2 to 4 following infection and then diminished in size and number (Fig. 4). Single-cell lysis reached significant levels following the period of maximal syncytium formation, on days 5 to 12 following infection. By days 11 to 13 following infection, Jurkat cells that expressed viral proteins but were resistant to cytopathic effect emerged. A similar pattern of viral protein expression and cytopathic effect has been observed previously in Jurkat cell cultures transfected with infectious HIV-1 proviral DNA (27).

It has been reported that the HIV-1 envelope glycoproteins in infected cells form complexes with the CD4 glycoprotein and that these complexes exist on the cell surface early in infection (18). Later in infection, the complexes are found primarily intracellularly, after which the absolute level of CD4 expression in the culture is significantly decreased (18, 27). The latter effect is perhaps secondary to viral cytopathic effect, which counterselects cells with high levels of CD4 expression.

To examine the time course of CD4 expression in the infected Jurkat cultures, fluorescence-activated cell sorting was performed at different time points following HIV-1 infection (Fig. 5). Two anti-CD4 monoclonal antibodies, OKT4 and OKT4A, were used in this analysis. The OKT4 antibody recognizes a CD4 epitope distant from the gp120 binding site, whereas the OKT4A antibody competes with gp120 for CD4 binding (34). On day 4 after infection of Jurkat cells with HIV-1 under these conditions, both the OKT4 and OKT4A epitopes of CD4 were detectable on the surface of the infected cultures. By day 5 following infection, the OKT4A antibody no longer recognized the surface of infected cultures. By contrast, the OKT4 epitope, although decreased in level relative to that observed at day 4 postinfection, remained accessible on days 5 to 9 following infection. These results are consistent with the presence of CD4 molecules saturated with the gp120 glycoprotein on the infected cell surface on days 5 to 9 postinfection. By day 13

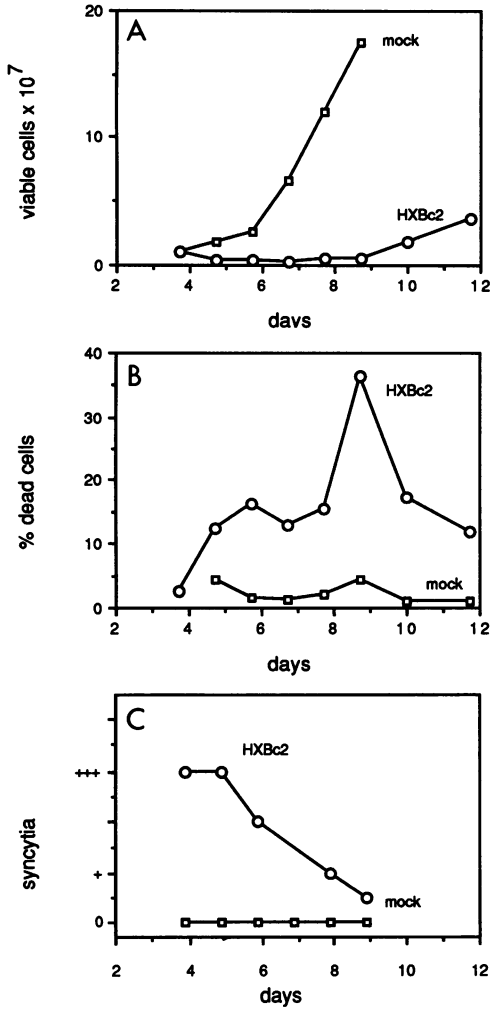

FIG. 4. Cytopathic effects of HIV-1 infection of Jurkat lymphocytes. (A) Effect of HIV-1 infection on the viability of the Jurkat lymphocytes infected with the HXBc2 strain of HIV-1 (○) compared with that of mock-infected Jurkat cells (□). (B and C) Percentage of single cells that stained positive with trypan blue (B) and the number of syncytia observed (C). Syncytia were scored as follows: +, 1 to 2 syncytia per 5 high-power (100×) fields in a culture of 10^6 cells per ml; +++, 15 to 25 syncytia per field; +++++, 40 to 50 syncytia per field.

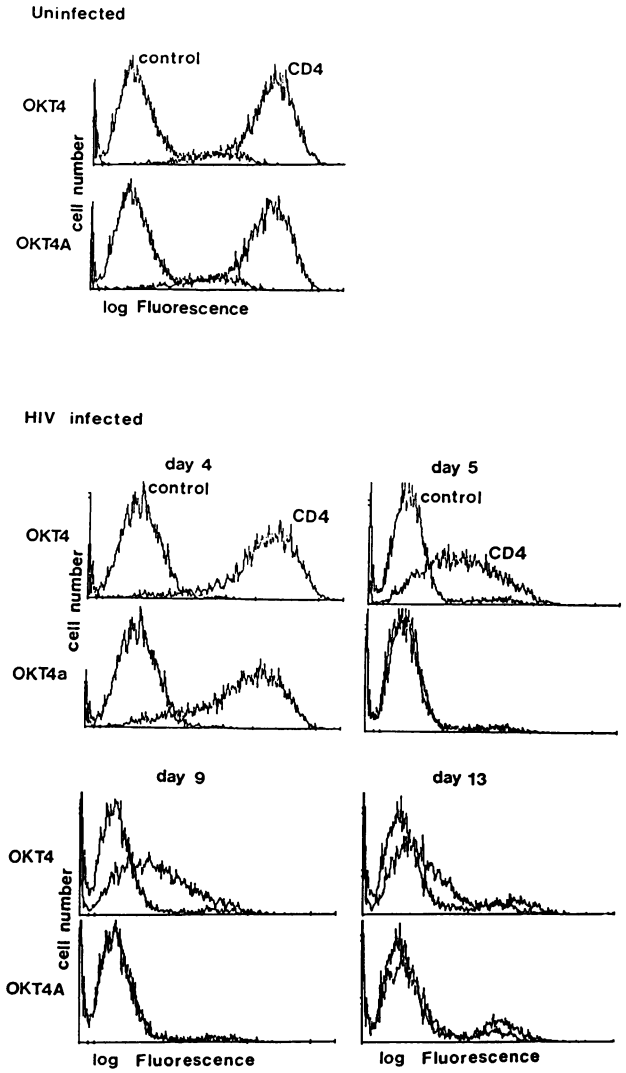

FIG. 5. Cell surface expression of CD4 in HIV-1-infected Jurkat cultures. Fluorescence-activated cell sorting was performed on either mock-infected or HXBc2-infected Jurkat lymphocytes with either the OKT4 or OKT4A monoclonal antibody, and then FITC-conjugated goat anti-mouse immunoglobulin was added. The control curve shows the results after treatment of cells with the second antibody alone. The time points represent days after infection with the HXBc2 virus.

following infection, cell surface CD4 was no longer detected by either the OKT4 or OKT4A antibody, consistent with both intracellular complexing and resultant decreased export of the envelope glycoprotein complexes as well as decreased absolute CD4 expression due to counterselection (18, 27). These results suggest that in the infected Jurkat cultures, free CD4 is available on the cell surface during the period of syncytium formation, consistent with the requirement for that process for CD4 (31, 47). During the phase of maximal single-cell lysis, most of the surface-accessible CD4 appears to be complexed to HIV-1 envelope glycoproteins. Finally, during the phase of emergence of cells resistant to HIV-1 cytopathic effect (day 11 and later), cell surface CD4 expression is minimal.

To assess the degree of superinfection occurring in the cultures, the level of unintegrated HIV-1 proviral DNA was assessed at different times following infection (Fig. 6). Unintegrated HIV-1 DNA representing linear and one- and two-LTR circular forms were observed, with maximal levels seen on day 2 following infection. The levels of linear unintegrated viral DNA, which represent preintegration forms, and the two-LTR circles decreased rapidly after day 2 postinfection, essentially disappearing by day 6 after infection. The level of the single-LTR circular form also decreased after day 2, but it was still detectable at day 6 after infection. Since the circular forms are derived from the linear unintegrated viral DNA (8, 46), this probably represents residual unintegrated DNA, since continued superinfection would be expected to generate greater amounts of linear than of circular proviral forms. No unintegrated viral DNA of any form was detected by 8 days after infection. These results suggest that in this system, the generation of

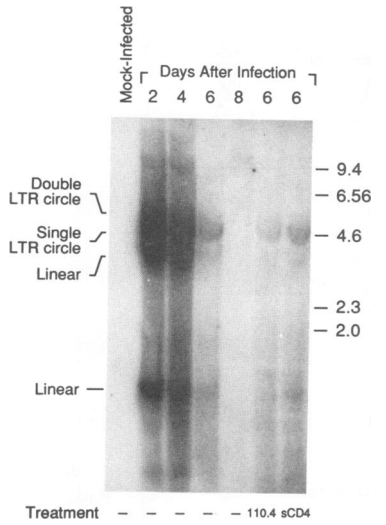

FIG. 6. Unintegrated viral DNA in HIV-1-infected Jurkat lymphocytes. Unintegrated viral DNA derived from Hirt supernatants observed in a Southern blot at various times following infection of Jurkat lymphocytes with the HXBc2 virus is shown. Jurkat lymphocytes were either untreated or treated with soluble CD4 (sCD4, 10 μg/ml, beginning at day 4 postinfection) or the 110.4 monoclonal antibody (20 μg/ml, beginning at day 4 postinfection). The positions of molecular size markers (in kilobase pairs) and expected fragment sizes are indicated.

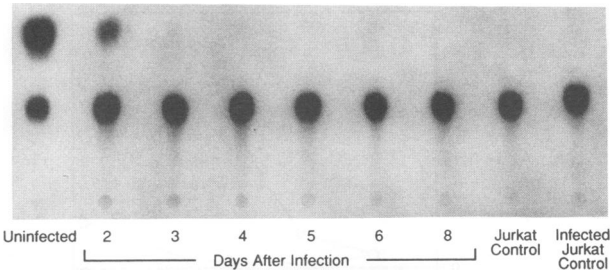

FIG. 7. Establishment of superinfection resistance in HIV-1-infected Jurkat cultures. The conversion of chloramphenicol to acetylated forms in Jurkat cells exposed to the HIV-CAT virus at different time points following infection with the HXBc2 virus is shown. The result with uninfected Jurkat target cells is shown for comparison with the results with infected Jurkat cells at 2 to 8 days postinfection. The background of CAT activity in uninfected Jurkat cells (Jurkat control) and HXBc2-infected Jurkat cells (infected Jurkat control) not exposed to the HIV-CAT virus is shown in the two right-hand lanes.

new unintegrated viral DNA occurs early following infection and is significantly attenuated by the time that single-cell lysis occurs.

The above results imply that superinfection occurs only for a limited, early period in the course of Jurkat cell infection. To assess the ability of the virus to superinfect the infected Jurkat cells directly, Jurkat cultures infected with the HXBc2 virus were exposed to a replication-competent HIV-1 encoding bacterial CAT (HIV-CAT). Infection of target cells by the HIV-CAT virus can be quantitatively assessed by measuring CAT activity in cell lysates. Two days after infection with the HXBc2 virus, the level of infection of Jurkat cells with the HIV-CAT virus was less than 12% of that of uninfected Jurkat cells (Fig. 7). At later times following HXBc2 infection, infection by the HIV-CAT virus could not be detected. These results agree with the data described above suggesting that superinfection resistance is established early (within 2 to 4 days) following infection of Jurkat cultures with the HXBc2 virus under these conditions. The period of superinfection corresponds to the phase of syncytium formation but significantly precedes the phase of single-cell lysis.

Effect of AZT treatment on single-cell lysis in Jurkat cells. The studies with C8166 lymphocytes described above suggests that although some antiviral agents could limit superinfection, little effect on single-cell lysis mediated by HIV-1 was observed. To examine the generality of these results, Jurkat lymphocytes infected with the HXBc2 virus were treated on day 3 following infection with 10 nM AZT. AZT was added at day 3 after infection because pilot experiments revealed that earlier addition of this agent caused a significant reduction in viral protein expression in the cultures (data not shown). The concentration of AZT used was determined by a single-step *env* complementation assay with Jurkat target cells (Materials and Methods).

The time course of cytopathic changes in this experiment is shown in Fig. 8. In untreated infected cultures, syncytium formation decreased by day 4 following infection and single-cell lysis reached significant levels on days 5 to 10 postinfection. The levels of unintegrated viral DNA were highest at day 2 after infection and showed some decrease by day 4, primarily in the linear and double-LTR forms (Fig. 9A). Addition of AZT to the Jurkat culture at day 3 after infection caused a dramatic reduction in the level of unintegrated viral DNA by the end of the following day (Fig. 9A). The level of viral protein expression, normalized to the number of viable cells, was not affected by AZT treatment (Fig. 9B). The AZT treatment did not affect the cytopathic effects of HIV-1 infection in the Jurkat cultures, as both syncytium formation and single-cell lysis occurred to the same degree in AZT-treated and untreated cultures (Fig. 8). In Jurkat cells, as in C8166 cells, a significant reduction in the accumulation of unintegrated HIV-1 DNA did not attenuate cytopathic effect.

Effects of anti-gp120 and anti-CD4 agents on single-cell lysis. To examine whether agents directed at the receptor-binding or membrane fusion functions of the HIV-1 envelope glycoproteins could inhibit single-cell lysis, soluble CD4 or the OKT4A or 110.4 monoclonal antibody was added to previously infected Jurkat cultures. Both soluble CD4 and the OKT4A antibody competitively inhibit the HIV-1 gp120-CD4 interaction by binding gp120 or CD4, respectively (7a, 9a, 19a, 34, 46c, 57). In addition, soluble CD4 has been reported to induce shedding of the HIV-1 gp120 glycoprotein (14, 23, 35, 36). The addition of 0.66 μg of the OKT4A antibody per ml was shown in other experiments to inhibit binding of radiolabeled gp120 to CD4-positive lymphocytes and to inhibit the infection of Jurkat cells by approximately 90%. Increasing the OKT4A concentration beyond 0.66 μg/ml did not increase the degree of virus neutralization in these pilot experiments (data not shown). As shown in Fig. 8, little effect on syncytium formation was observed after OKT4A addition to HIV-1-infected Jurkat cells, since in this experiment the number of syncytia were already decreasing at the time of OKT4A addition. The addition of OKT4A did not significantly alter the levels of unintegrated viral DNA (Fig. 9A) or viral proteins (Fig. 9B). A slight inhibitory effect on single-cell lysis was observed (Fig. 8).

The failure of the OKT4A monoclonal antibody to signif-

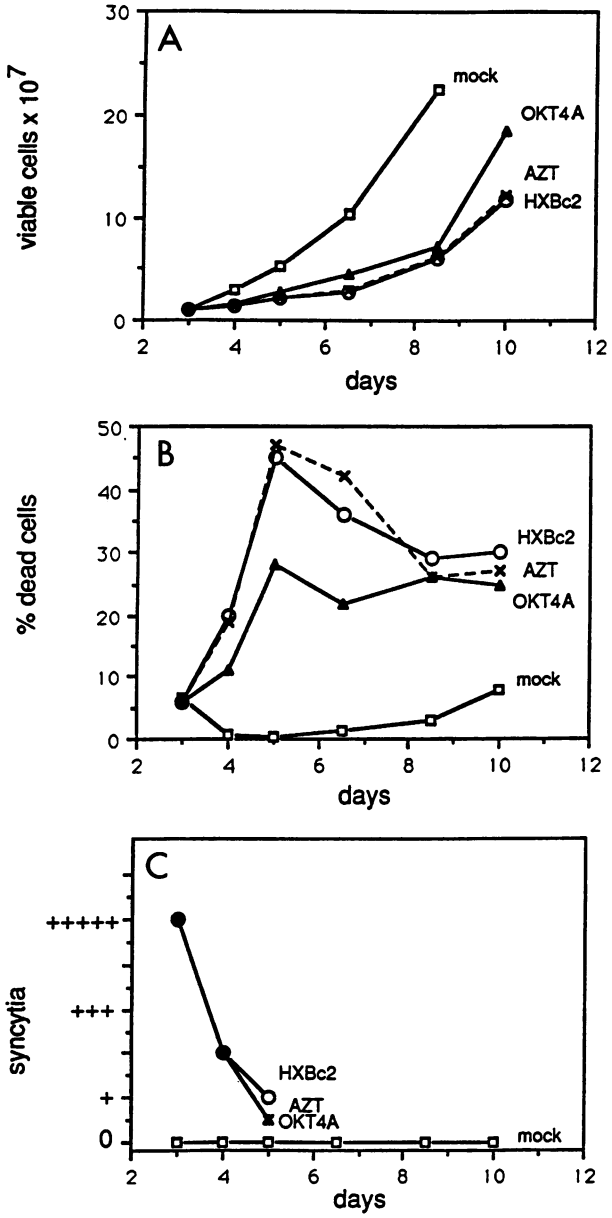

FIG. 8. Effect of AZT and OKT4A antibody on HIV cytopathic effect in Jurkat cells. The cytopathic effects of HXBc2 infection of Jurkat lymphocytes are shown, with scoring of cell viability (A), single-cell lysis (B), and syncytium formation (C) done as described in Fig. 1 legend and in Materials and Methods. Infected cultures were either untreated (○) or treated with AZT (10 nM, beginning at day 3 post-infection) (×) or OKT4A monoclonal antibody (0.33 μg/ml, beginning at day 3 post-infection) (▲). □, mock-infected cultures.

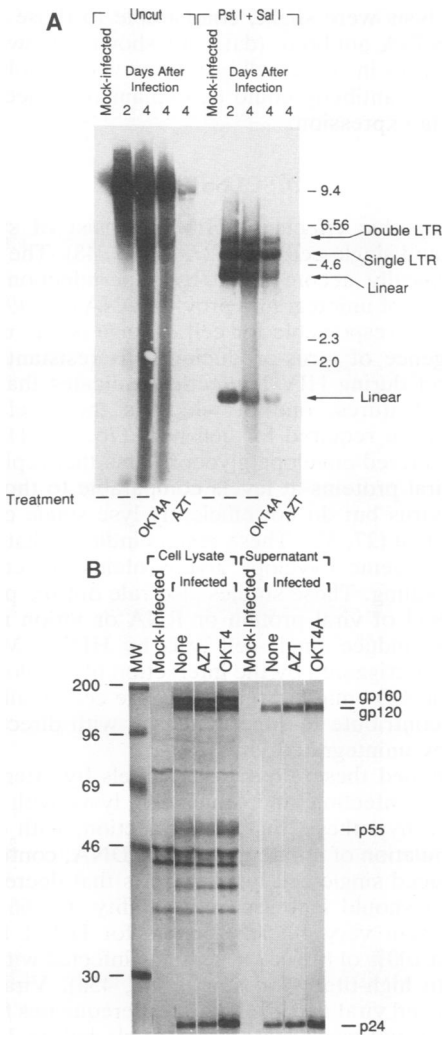

FIG. 9. Effects of AZT and OKT4A antibody treatment on viral DNA and protein expression. HXBc2-infected Jurkat cultures were treated as described in the legend to Fig. 8. (A) Viral DNA derived from Hirt supernatants, either uncut or digested with *PstI* plus *SalI*, was assessed by Southern blotting at the indicated times after HXBc2 infection. Sizes are shown in kilobase pairs. (B) Viral protein expression in 5×10^5 viable cells was assessed by radioimmunoprecipitation of either cell lysates or supernatants. The particular viral proteins are identified to the right of the figure. Sizes are shown in kilodaltons.

icantly inhibit single-cell lysis by HIV-1 may be explained by the relative unavailability of the epitope at the time of single-cell killing, probably due to saturation of cell surface CD4 with envelope glycoproteins. For HIV-1 and other cytopathic retroviruses, neutralizing antibodies directed against the viral envelope glycoproteins have been reported to inhibit viral cytopathic effect, although in most cases, whether such treatment indirectly affects cytolysis by decreasing overall levels of viral protein expression has not been clearly addressed (21, 38, 59). To examine this, soluble CD4 or the 110.4 antibody was added to Jurkat cultures 4 days following infection with the HXBc2 virus. Addition of these agents earlier than 4 days after infection invariably caused a decrease in total viral protein expression in the infected Jurkat cultures (data not shown). Even when these agents were added at 4 days postinfection, subtle decreases in viral protein expression were detected by immunoprecipitation of infected cells (data not shown). Neither soluble CD4 nor 110.4 antibody treatment initiated on day 4 following infection resulted in decreased levels of unintegrated HIV-1 DNA (Fig. 6). This was expected because, at the time of addition of the agents, the levels of unintegrated viral DNA were already declining without treatment. The effects of soluble CD4 and 110.4 monoclonal antibody treatment on

single-cell lysis were slight, comparable to those observed for the OKT4A antibody (data not shown). However, the slight decreases in single-cell lysis observed for soluble CD4 and the 110.4 antibody could be explained by decreases in viral protein expression.

DISCUSSION

The cytopathic effects of HIV-1 consist of syncytium formation and single-cell lysis (27, 31, 47, 48). The killing of cells is typically accompanied by superinfection and the accumulation of unintegrated proviral DNA (43, 49), but the exact process responsible for cell damage is still unknown. The emergence of virus-producing cells resistant to cytopathic effect during HIV-1 infection indicates that specific target cell features, one of which is the level of CD4 expression, are required for cell lysis (7b, 27). HIV-1 mutants with altered envelope glycoproteins that replicate and produce viral proteins at levels comparable to those of the wild-type virus but do not efficiently lyse single cells have been identified (27, 52). These results indicate that properly cleaved, fusogenic envelope glycoproteins are critical for single-cell killing. These studies also rule out the possibility that the level of viral protein or RNA or virion release is sufficient to induce single-cell lysis by HIV-1. Membrane fusion events triggered by the interaction of envelope glycoproteins and CD4 might directly damage cell membranes or indirectly contribute to superinfection, with direct toxicity mediated by unintegrated viral DNA.

We examined these alternative models by attempting to modify superinfection and single-cell lysis with antiviral agents. The hypothesis that superinfection, with concomitant accumulation of unintegrated viral DNA, contributes to HIV-1-induced single-cell lysis predicts that decreasing superinfection should increase cell viability. C8166 lymphocytes represent very efficient targets for HIV-1 infection, with almost 100% of the cells becoming infected within 6 h of exposure to high-titer viral stocks (43, 45a). Viral protein production and viral particle release, prerequisites for superinfection, do not reach detectable levels before 12 h after exposure to virus (43). The efficient initial infection of C8166 cells, combined with the delay between the initial and subsequent rounds of infection, makes this system ideal for examining the effects of blocking superinfection. Treatment of infected C8166 cells with the 110.4 neutralizing antibody at 8 h postinfection dramatically curtailed superinfection, as evidenced by an almost complete absence of unintegrated HIV-1 DNA. This decrease in superinfection was accompanied by a small decrease in levels of viral protein expression and minimal decreases in single-cell lysis. These results suggest that superinfection and accumulation of unintegrated viral DNA are not necessary for the lysis of single cells by HIV-1. However, superinfection may, by increasing the level of viral protein expression, contribute to the efficiency of cytolysis. This auxiliary role of superinfection in viral cytopathic effect appears to be at least partly operative even in C8166 cells, which produce human T-cell leukemia virus type I regulatory proteins that are known to increase HIV-1 gene expression (41a, 46a). The contribution of superinfection to HIV-1 protein expression in cells lacking human T-cell leukemia virus type I proteins would be expected to be at least as great as that observed in the C8166 cell line. Estimating this contribution in other cell types is more difficult because the efficiency of initial infection in these cells is lower than in C8166 cells. Consequently, antiviral agents may block, in addition to superinfection, the de novo infection of previously uninfected cells that contributes to total viral protein expression in the culture.

Treatment of infected C8166 and Jurkat cells with AZT significantly reduced the generation of unintegrated viral DNA but affected neither syncytium formation, single-cell lysis, nor viral protein expression. These results support the conclusion that the generation of high levels of unintegrated viral DNA is not necessary for single-cell killing by HIV-1. In addition, the results imply that some fraction of the unintegrated viral DNA present in HIV-1-infected cells does not contribute to viral protein expression. It is likely that the contribution of superinfection to viral protein expression is greater when the number of viral DNA copies per cell is small.

The temporal relationship between superinfection and single-cell lysis was documented in HIV-1-infected Jurkat lymphocytes. In this system, the OKT4A epitope on CD4, which overlaps the gp120-binding site (34), was inaccessible by 4 to 5 days following infection, presumably because of complex formation with the HIV-1 envelope glycoproteins. Based on the levels of unintegrated viral DNA and on the resistance of cells to infection with an exogenous virus carrying the CAT gene, interference and superinfection resistance were established within 4 days postinfection. During this early postinfection period, syncytium formation was the prominent cytopathic effect observed. As the number of gp120-accessible CD4 molecules on the cell surface diminished, syncytium formation ceased, consistent with the requirement for cell surface CD4-envelope glycoprotein binding for this process (31, 47). The disappearance of unintegrated viral DNA at this time could result in part from the demise of syncytia, which would be expected to be efficient targets for superinfection. Single-cell lysis, by contrast, was minimal during the peak period of superinfection and reached significant levels several days after interference and superinfection resistance had already been established. This result suggests that if superinfection contributes to single-cell lysis in the Jurkat cells, there must be a significant temporal delay between cause and effect. This temporal pattern would be more consistent with superinfection indirectly contributing to cell lysis by augmenting proviral copy number and viral protein expression rather than with superinfection participating directly in the cytopathic process.

Our results indicate that single-cell killing of Jurkat lymphocytes by HIV-1 occurs at a time when the vast majority of cell surface CD4 is complexed with envelope glycoproteins and when the level of superinfection is minimal. In both the Jurkat and C8166 systems, soluble CD4 or antibodies directed against the gp120 or CD4 glycoprotein had minimal effects on single-cell lysis, and most of the observed effects could be attributed to subtle decreases in the levels of viral protein expression. It is possible that soluble CD4 and OKT4A antibody fail to significantly affect single-cell lysis because most of the relevant damage is mediated by envelope glycoprotein-CD4 complexes that are formed prior to export to the cell surface. One would expect that the 110.4 antibody should block membrane fusion events at the cell surface even for such preformed envelope glycoprotein-CD4 complexes. The apparent absence of direct effects of 110.4 antibody treatment on single-cell lysis suggests that the majority of envelope glycoprotein-mediated events that contribute to single-cell killing may occur in cell compartments not accessible to large proteins such as soluble CD4 and antibodies. Significant levels of intracellular envelope glycoprotein-CD4 complexes have been observed in HIV-1-infected cells and in cells coexpressing these glycoproteins

(18, 20, 26, 53). This could provide a context in which fusion events lead to membrane damage and, ultimately, loss of cell viability. Other proposed models, such as that envelope glycoprotein-CD4 complexes lead to blocking of nuclear pores (25), do not explain the requirement for cleavage-activated, fusogenic envelope glycoproteins for single-cell lysis. In the systems used by Koga et al. (25, 26), single-cell death is observed with almost exclusive expression of the gp160 envelope glycoprotein precursor, in the presence of cadmium, in CD4-positive lymphocytes. The observed defects in the integrity of nuclear pores (25) have not been described in lymphocytes undergoing lysis during the course of HIV-1 infection.

Although no obvious relationship between superinfection and single-cell lysis could be documented in this work, envelope glycoprotein-receptor interactions occurring in the context of viral superinfection might be important for killing of host cells by retroviruses other than HIV-1. The observation that decreased envelope glycoprotein precursor processing is associated with increased cytopathic effect for feline leukemia viruses (39) but decreased single-cell lysis by HIV-1 (52) suggests that the specific context of envelope glycoprotein-related events critical for cell killing may differ for different viruses.

Recently, cellular DNA fragmentation, interpreted to indicate the presence of apoptosis, has been observed in HIV-1-infected cultures (54). In the Jurkat cell system used herein, cellular DNA fragmentation was occasionally and transiently observed early (within 2 to 3 days) after infection, but was not consistently associated with the period of single-cell lysis. No cellular DNA fragmentation was observed in the infected C8166 cultures examined at 2 to 3 days following infection. The cellular DNA fragmentation reported for other systems probably represents a secondary consequence of cell death and does not imply a specific mechanism of cell destruction by HIV-1.

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