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Molecular Determinants of Acute Single-Cell Lysis by Human Immunodeficiency Virus Type 1

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Human immunodeficiency virus type 1 (HIV-1) infection of CD4-positive lymphocytes is accompanied by acute cytopathic effects, i.e., syncytium formation and single-cell lysis. Syncytium formation involves cell-cell fusion mediated by viral envelope glycoproteins on the surface of infected cells and by CD4 glycoproteins on adjacent cells. The molecular basis for the lysis of single HIV-1-infected cells is unclear. Here we report that the expression of functional envelope glycoproteins from primary and laboratory-adapted HIV-1 isolates resulted in the lysis of single CD4-positive lymphocytes. As was previously observed in HIV-1-infected cultures, single-cell lysis in this system primarily involved necrosis and was not inhibited by soluble CD4. Binding of the viral envelope glycoproteins to the CD4 glycoprotein facilitated, but was not sufficient for, cytolysis. Importantly, the ability of the HIV-1 envelope glycoproteins to mediate membrane fusion was essential for single-cell killing. By contrast, the long cytoplasmic tail of the gp41 transmembrane envelope glycoprotein was neither necessary nor sufficient for single-cell lysis. These results suggest that intracellular envelope glycoprotein-CD4 interactions initiate autofusion events that disrupt cell membrane integrity, leading to single-cell lysis by HIV-1.

Human immunodeficiency virus type 1 (HIV-1) establishes persistent infections in humans, in most cases leading to the development of AIDS (2, 22, 28). HIV-1 infects CD4-positive lymphocytes, monocytes, and some subsets of dendritic cells, in both the peripheral blood and lymphoid organs (23, 66). HIV-1 infection is accompanied by the progressive loss of CD4-positive lymphocytes, leading to immunodeficiency (22, 23). Studies with HIV-1-infected humans suggest a relationship between the viral burden and the slope of CD4-positive lymphocyte decline (7, 14, 34, 64). Recent studies of viral and lymphocyte dynamics indicate that in contrast to latently infected cells, which exhibit normal life spans, HIV-1-producing cells exhibit in vivo half-lives of only 2 to 3 days (35, 79). This rapid destruction of virus-producing lymphocytes increases progressively as the duration of infection and the viral burden increase. The destruction of virus-producing lymphocytes is only partially compensated by lymphocyte production, leading to a slow, progressive decline in the steady-state level of CD4-positive lymphocytes.

Two mechanisms that are likely to contribute to the rapid in vivo destruction of cells producing HIV-1 are viral cytopathic effect and immunologic clearance (35, 79). Depending upon particular circumstances, both of these mechanisms probably account for the destruction of some HIV-1-producing cells. Because the efficiency of immunologic clearance is itself dependent upon the number of CD4-positive cells, this mechanism alone is insufficient to account for the observed inexorable decline in CD4 lymphocytes in most HIV-1-infected people. Furthermore, recent studies suggest that individuals exhibiting inefficient cytotoxic immune responses capable of lysing HIV-1-infected cells demonstrate more rapid rates of CD4 lymphocyte decline (46). These considerations make it likely that the HIV-1 cytopathic effect contributes at least partially to the destruction of CD4 lymphocytes in vivo.

Cytopathic effects typically occur within 1 or 2 weeks following HIV-1 infection of cultured lymphocytes and are most prominent in cells expressing high levels of the CD4 glycoprotein (18), which acts as the receptor for the virus (15, 40). HIV-1 cytopathic effects consist of the formation of multinucleated giant cells (syncytia) and the lysis of single cells (67, 71). Syncytium formation is mediated solely by the HIV-1 envelope glycoproteins, gp120 and gp41, expressed on the infected-cell surface (52, 69). The gp120 exterior envelope glycoprotein binds to the CD4 receptor on adjacent cells, and then, via a fusion reaction analogous to that involved in virus entry, the apposed cell membranes are fused so that heterokaryons are formed. The hydrophobic amino terminus of the gp41 transmembrane envelope glycoprotein, which exhibits sequence similarity to the fusion peptides of other enveloped viruses, has been shown to play a critical role in the membrane fusion process (26, 33, 48). By contrast, the unusually long gp41 cytoplasmic tail is dispensable for syncytium formation (48, 78, 81).

Single-cell lysis accounts for the destruction of most HIV-1-infected cells in tissue culture (71). Unlike syncytium formation, single-cell lysis is not susceptible to inhibition by soluble CD4 or neutralizing antibodies (3). In light of the substantial concentrations of neutralizing antibodies present in the sera of most HIV-1-infected individuals, the contribution of single-cell lysis to the in vivo destruction of infected cells may be greater than that of syncytium formation. While some syncytia formed in HIV-1-infected cultures undergo apoptosis (51, 51a, 77), the percentage of dying single cells that exhibit apoptotic changes may be very low (<5%), depending upon the cell line used (4, 65). These observations suggest that apoptosis is not a universal feature of cells dying from HIV-1 infection and probably does not represent a necessary pathway for HIV-1-induced cell lysis.

The molecular mechanism of single-cell lysis by HIV-1 is still unresolved. Experiments with genetically altered HIV-1 viruses have demonstrated that the viral regulatory proteins (Vif, Vpr, Tat, Vpu, and Nef) are not responsible for the acute lysis

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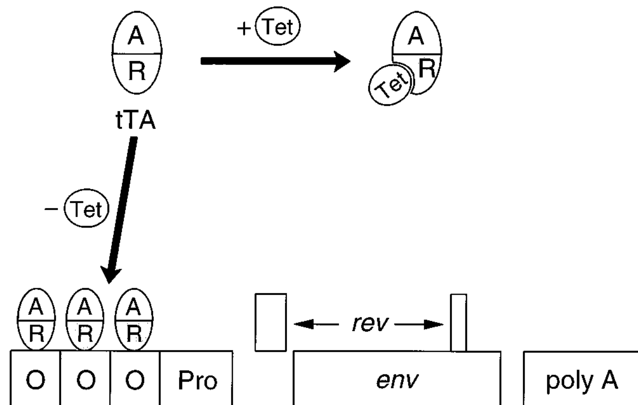

FIG. 1. System for inducible expression of the HIV-1 envelope glycoproteins. The binding of the tTA protein, which contains the VP16 activator (A) fused to the *tet* repressor (R), to the *tet* operator (O) sequences upstream of the minimal cytomegalovirus promoter (Pro) is inhibited by tetracycline (Tet). Polyadenylation signals (poly A) from simian virus 40 are shown.

of single cells observed upon HIV-1 infection (16, 68, 70). Studies of other mutant HIV-1 viruses have implicated the viral envelope glycoproteins in single-cell lysis. A gp120 amino acid change affecting the efficiency of processing of the gp160 envelope glycoprotein precursor was reported to decrease the ability of a replication-competent HIV-1 virus to lyse single cells (73). An insertion of four amino acids into the gp41 amino terminus resulted in a partial decrease in membrane fusion activity. Viruses containing this change replicated and expressed high levels of viral proteins in infected cells but were attenuated in the abilities to form syncytia and to lyse single cells (47). These studies imply that a fusogenic function of the envelope glycoproteins, which requires an intact gp41 amino terminus (26, 48) and a cleaved gp160 precursor (56), contributes to the efficient lysis of single cells by HIV-1. Here, we tested this hypothesis by expressing wild-type and mutant HIV-1 envelope glycoproteins in an inducible fashion in CD4-positive lymphocytes.

MATERIALS AND METHODS

Construction of plasmids. The tetracycline-controlled transactivator (tTA)-expressing plasmid pUHD15-1 and its response plasmid pUHD10-3 were made and kindly provided by Manfred Gossen and Hermann Bujard at the University of Heidelberg, Heidelberg, Germany. The construction of these plasmids has been described elsewhere (30). In brief, the regulator plasmid pUHD15-1, encoding tTA, contains the human cytomegalovirus promoter-enhancer, the tTA coding sequences, and a simian virus 40 polyadenylation signal. tTA is a fusion protein combining the tetracycline repressor (TetR) with the C-terminal domain of VP16 from herpes simplex virus (Fig. 1). To establish a cell line constitutively expressing tTA, we constructed the pUHD15-1neo plasmid, which contains a neomycin resistance cassette inserted at the *Xho*I site. The neomycin resistance cassette (about 2.7 kb), containing an SL3-3 long terminal repeat and a simian virus 40 polyadenylation signal, was derived from the pSU3T4pANeo plasmid (11). The response plasmids, pUHD10-3 and its derivatives, have a tTA-dependent promoter containing tetracycline operator sequences (TetO) linked to a human cytomegalovirus minimal promoter (30). The reporter plasmid, pUHD10-3CAT, was constructed by inserting a chloramphenicol acetyltransferase (CAT) gene (derived from pCM4 [Pharmacia]) at a *Bam*HI site within the multiple cloning sites of pUHD10-3. The HIV-1 *env*-expressing plasmids (pUHD10-3hygENV plasmids) were constructed by inserting hygromycin resistance genes, driven by a thymidine kinase promoter (derived from p3′SS [Stratagene]), at the *Xho*I site of pUHD10-3, yielding pUHD10-3hyg. The HIV-1 *env* genes, including wild-type (strain HXBc2, 89.6, and ADA) and mutant (strain HXBc2) genes, were recovered from pSVIIIenv (63, 75) by *Sal*I and *Xho*I digestions. The *Sal*I-*Xho*I fragments, which contain the HIV-1 *rev* and *env* genes, were cloned into pUHD10-3hyg at the *Bam*HI site within the multiple cloning site by the partial filling in of cleaved termini.

Establishment of stable cell lines. Jurkat, a human CD4-positive lymphocyte line, was transfected with 10 μg of linearized pUHD15-1neo by electroporation. Briefly, 5 × 10^6 cells in 0.5 ml of serum-free RPMI 1640 medium were mixed with 10 μg of DNA, incubated on ice for 10 min, and electroporated at 270 V and 960 μF by using a Bio-Rad Gene Pulser unit. After electroporation, the cells were selected with 800 μg of G418 (Gibco BRL) per ml for 2 to 3 weeks. The transfectants that were resistant to G418 were isolated and cloned by the limiting-dilution method. The tTA expression in these selected clones was examined by a functional assay using pUHD10-3CAT. Briefly, 4 × 10^6 cells from each clone were transfected with 1.25 μg of pUHD10-3CAT, and half of these cells were cultured in either the presence or absence of 1 μg of tetracycline per ml. Three days after transfection, CAT activity was measured as described previously (32). The clone (D4) that exhibited the highest level of CAT activity in the absence of tetracycline and that exhibited the lowest level of CAT activity in the presence of tetracycline was chosen as a host cell for transfection with each of the HIV-1 *env* expression plasmids (pUHD10-3hygENV plasmids). Such transfected cells were selected in RPMI 1640 medium containing 10% fetal bovine serum, 200 μg of G418 per ml, 200 μg of hygromycin (Boehringer Mannheim) per ml, and 1 μg of tetracycline (Sigma) per ml for 2 to 3 weeks. The hygromycin-resistant clones were isolated by limiting dilution and screened by radioimmunoprecipitation with AIDS patient serum 3 days following induction by removal of tetracycline from the medium. The clones expressing the highest levels of HIV-1 envelope glycoproteins were selected for further studies. The selected clones were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 200 μg of G418 per ml, 100 μg of hygromycin per ml, and 1 μg of tetracycline per ml in a 37°C incubator with 5% CO_2.

Induction of HIV-1 envelope glycoprotein expression and radioimmunoprecipitation. To induce the selected clones to express HIV-1 envelope glycoproteins, cells were washed with phosphate-buffered saline (PBS) and cultured in RPMI 1640 medium as described above but without tetracycline in the medium. Three days following induction, 5 × 10^6 cells were labeled with 50 μCi of [^{35}S]cysteine per ml for 12 h and precipitated with a mixture of sera from HIV-1-infected individuals.

For titration of envelope glycoprotein expression, the cells from clones HXBc2(4) and ADA(2) were cultured in medium with various concentrations of tetracycline (0, 1, 2.5, 5, 10, 100, and 1,000 ng/ml). After 3 days of incubation, 2 × 10^6 cells from each clone were labeled with 50 μCi of [^{35}S]cysteine per ml and precipitated with a mixture of sera from HIV-1-infected individuals. The immunoprecipitated HIV-1 envelope glycoproteins were separated on sodium dodecyl sulfate–7.5% polyacrylamide gels and visualized by autoradiography. The relative intensity of the band corresponding to the gp120 glycoprotein was measured by densitometry (Digital Imaging Systems IS-1000).

Measurement of cell surface CD4 expression. Cell surface CD4 expression was measured by fluorescence-activated cell sorter (FACS) analysis with a monoclonal antibody, OKT4 (Ortho Diagnostic Systems), which recognizes an epitope distant from the envelope glycoprotein-binding site (57, 58) and a fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G monoclonal antibody (Sigma). Approximately 10^6 cells, cultured in either the presence or absence of 1 μg of tetracycline per ml for 3 to 4 days, were incubated with 100 μl of OKT4 antibody (1:33 dilution) for 30 min on ice and then washed twice with cold PBS containing 2% fetal bovine serum. To these cells, 100 μl of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G antibody (1:100 dilution) was added. After being incubated and washed as described above, the cells were fixed in 2% formaldehyde in PBS and analyzed by flow cytometry (Becton Dickinson FACScan). The median of fluorescence intensity was calculated as an indicator of the level of cell surface CD4 expression.

Measurement of cytopathic effect. To assess the cytopathic effect mediated by the HIV-1 envelope glycoproteins, 2 × 10^6 cells from each clone were harvested and washed once in PBS, and half of the cells were grown in either the presence or absence of 1 μg of tetracycline per ml. To block syncytium formation following induction of envelope glycoprotein expression, soluble CD4 (AGMED) (37) was added to the cultures of clones HXBc2(6), 713 STOP, and 89.6(6) at a concentration of 10 μg/ml. Soluble CD4 was not added in the studies designed to examine the formation of syncytia. Cultures were replenished with fresh medium (half of the original volume) every 2 days. The cytopathic effect was measured every 2 days by counting the number of syncytia, the percentage of trypan blue-stained single cells, or the total number of viable cells not stained by trypan blue dye (3, 6, 47). These experiments were repeated at least three times, and the results of a typical set of experiments are presented here.

Detection of apoptosis. At various times following induction of envelope glycoprotein expression, the relative numbers of apoptotic cells in the cultures of clones HXBc2(4) and ADA(2) were determined by using three methods: (i) Wright-Giemsa staining, (ii) TdT-mediated dUTP nick end labeling (TUNEL), and (iii) assessment of DNA fragmentation.

(i) Wright-Giemsa staining. Cells expressing the HXBc2 or ADA envelope glycoproteins were harvested at 0, 3, 6, 9, and 12 days after induction and Wright-Giemsa stained (83). Apoptotic cells were identified by morphologic changes, including a decrease in cell volume, chromatin condensation, and the formation of apoptotic bodies (19). The percentage of apoptotic cells was scored by counting a total of 200 stained cells under a light microscope.

(ii) TUNEL. The fluorescence in situ cell death detection kit (Boehringer Mannheim) was used to examine clones HXBc2(4) and ADA(2) at 0, 5, and 10

days following the induction of envelope glycoprotein expression, according to the manufacturer's instructions. The same total number of cells from each sample was examined. Positive controls were prepared by treating the cells with 5 μM staurosporine (Sigma) (38) for 5 h at 37°C. In the TUNEL assay, cells that contain fragmented DNA with free 3′-OH ends are labeled with fluorescein and can be detected by flow cytometry (29). Briefly, 1.5×10^6 cells were washed with cold PBS (containing 2% bovine serum albumin), fixed with paraformaldehyde solution (4% in PBS, pH 7.5), and permeabilized with 0.1% Triton X-100 (in 0.1% sodium citrate). The cells were washed twice with PBS and incubated in 50 μl of TUNEL reaction mixture at 37°C for 1 h in the dark. After being washed twice with PBS, the labeled cells were analyzed by flow cytometry.

(iii) DNA fragmentation assay. The presence of fragmented DNA in the cultures of clones HXBc2(4) and ADA(2) at 0, 5, and 10 days following induction of envelope glycoprotein expression was investigated by the method of Kondo et al. (45). Positive control cells were prepared by staurosporine treatment as described above. In brief, total DNA was extracted from cells that were lysed and digested with proteinase K at 50°C for 12 h and was precipitated with ethanol-ammonium acetate. After incubation with 100 μg of RNase A per ml, the total cellular DNA was electrophoresed on a 2% agarose gel containing 0.5 μg of ethidium bromide per ml.

Electron microscopy. The cells harvested from the culture of clone HXBc2(4) at 0, 3, and 6 days following induction of envelope glycoprotein expression were fixed with 2.5% glutaraldehyde in PBS for 1 h at 4°C. After fixation, the cell pellets were washed with Sabatini's solution (PBS with 6.8% sucrose). The samples were postfixed with 1% osmium tetroxide, dehydrated in graded alcohols, and treated with propylene oxide. After embedment, ultrathin sections were cut with an MT2 Sorvall ultramicrotome, stained with lead citrate and uranyl acetate, and examined with a JEOL 100CX transmission electron microscope.

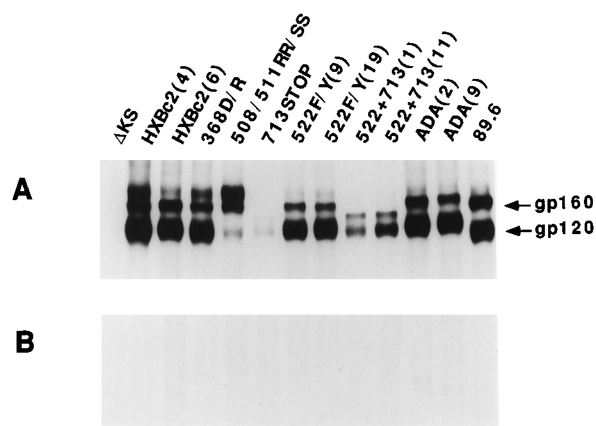

FIG. 2. Expression of the HIV-1 envelope glycoproteins in the Jurkat clones in the absence (A) or presence (B) of tetracycline (1 μg/ml), following precipitation with a mixture of sera from HIV-1-infected individuals. The positions of the precursor (gp160) and processed (gp120) glycoproteins are shown. Numbers in parentheses following some of the envelope glycoproteins indicate the individual clone of Jurkat cells used.

RESULTS

Inducible expression of HIV-1 envelope glycoproteins in Jurkat lymphocytes. Several HIV-1 envelope glycoproteins were selected to test whether the expression of these proteins in human CD4-positive lymphocytes can result in the lysis of single cells. The envelope glycoproteins derived from two primary clinical HIV-1 isolates, 89.6 and ADA, were chosen to represent each of the reported phenotypes of HIV-1 (1, 9, 24, 25). Some HIV-1 viruses, such as 89.6, replicate efficiently in peripheral blood lymphocytes ("rapid/high" phenotype) and are highly cytopathic (13, 75). Other primary isolates, such as ADA, replicate less efficiently in peripheral blood lymphocytes ("slow/low" phenotype) and are less cytopathic (75, 80). These phenotypic differences are determined by the exterior envelope glycoprotein, gp120 (8, 62). We also included the envelope glycoproteins from a laboratory-adapted HIV-1 strain, HXBc2, that is highly cytopathic for peripheral blood lymphocytes and for established T-lymphocyte lines (6, 47).

An inducible system based on the tetracycline operon was used to express the HIV-1 *env* genes in Jurkat human CD4-positive lymphocytes, which are susceptible to HIV-1 cytopathic effects (3, 6, 47). The *env* genes were expressed under the control of a cytomegalovirus minimal promoter fused to *tet* operator sequences (Fig. 1). The expression plasmid also encodes the HIV-1 Rev protein, which is required for efficient export of *env* messenger RNAs from the nucleus (70). A control plasmid (ΔKS) containing a deletion and frameshift within the HXBc2 *env* gene expresses only the HIV-1 Rev protein (4, 63). Jurkat lymphocytes constitutively expressing the chimeric tTA protein, which contains the *tet* repressor fused to the activation domain from the herpes simplex virus VP16 carboxyl terminus (30), were established. These cells were then transfected with the expression plasmids encoding the different HIV-1 *env* genes, and clones were selected in the presence of tetracycline, which suppresses envelope glycoprotein expression. To assess HIV-1 envelope glycoprotein expression in the Jurkat clones, each clone was cultured in the presence and absence of tetracycline for 3 days and then metabolically labeled. Labeled cell lysates were precipitated with an excess of a mixture of sera from HIV-1-infected individuals. The recognition of a variety of different epitopes on the envelope glycoproteins by these sera minimizes the effects of HIV-1 strain variation on the efficiency of immunoprecipitation (84). For each *env* variant, at least two independent clones were selected for detailed analysis, on the basis of the level of envelope glycoprotein expression. Figure 2 shows that comparable levels of expression of the 89.6, ADA, and HXBc2 envelope glycoproteins were attained in selected clones. These levels of envelope glycoprotein expression were comparable to those seen in Jurkat cultures 7 days after infection with the HXBc2 HIV-1 isolate, at a time when virus production peaks (data not shown). Envelope glycoprotein expression reached maximal levels by 3 days after induction and did not increase further thereafter (data not shown).

The level of CD4 expression on the surface of the Jurkat clones, in the presence and absence of tetracycline, was examined. FACS analysis was performed with the OKT4 monoclonal antibody, which recognizes a CD4 epitope unaffected by the binding of the HIV-1 envelope glycoproteins (57, 58). Table 1 shows that in the presence of tetracycline, the Jurkat clones containing the HXBc2 *env* genes exhibited the two extremes of CD4 expression, with clone 4 having a low level and clone 6 having a very high level. In the presence of tetracycline, CD4 expression on the Jurkat clones with the 89.6 and ADA *env* genes fell between these extremes. When the Jurkat clones were cultured in the absence of tetracycline to induce envelope glycoprotein expression, a decrease in the level of cell surface CD4 was seen in the clones expressing the 89.6, ADA, and HXBc2 envelope glycoproteins. No decrease in surface CD4 expression was seen when a control clone containing the ΔKS plasmid, which expresses only the HIV-1 Rev proteins, was cultured in the absence of tetracycline.

The growth and viability of the Jurkat clones in the presence and absence of tetracycline were examined (Fig. 3). In the presence of tetracycline, the growth of all of the Jurkat clones was equivalent (Fig. 3A). In the absence of tetracycline, the growth and appearance of the ΔKS clone, expressing only the Rev protein, were similar to those seen in the presence of tetracycline. By contrast, massive syncytium formation was observed in the Jurkat clone expressing the 89.6 envelope glycoproteins and in clone 6 expressing the HXBc2 envelope glycoproteins (Fig. 3B and Table 1). The number of syncytia peaked at 4 days after the induction of envelope glycoprotein expres-

TABLE 1. Characteristics of selected envelope glycoprotein-expressing Jurkat clones

Jurkat clone	Cell surface CD4 level[a]		Syncytia[b]	% Dead cells[c]
	With tetracycline	Without tetracycline		
ΔKS	8.6	9.1	0	3.1
89.6(6)	18.6	6.7	450	28.6
ADA(2)	6.9	4.7	0	2.5
ADA(9)	13.0	8.0	ND[d]	3.3
HXBc2(4)	5.7	3.6	56	26.7
HXBc2(6)	56.2	10.5	520	26.9
368 D/R	20.4	21.1	0	14.6
508/511 RR/SS	14.5	3.8	0	10.4
522 F/Y(9)	12.2	ND	ND	4.2
522 F/Y(19)	15.9	8.7	0	4.0
713 STOP	23.1	6.7	580	29.6
522+713(1)	5.7	ND	ND	7.0
522+713(11)	29.7	ND	ND	2.9

[a] The cell surface CD4 level (median fluorescence intensity) was measured as described in Materials and Methods.

[b] The number of syncytia per milliliter was counted from 2 to 6 days following transfer of clones into medium without tetracycline and without soluble CD4. The maximum number of syncytia observed during this period is reported.

[c] The percentage of single cells that were trypan blue positive was scored from 2 to 12 days following transfer of Jurkat clones into medium lacking tetracycline. Soluble CD4 was added to cultures of Jurkat cells expressing envelope glycoproteins with syncytium-forming ability. The maximum percentage of dead cells observed during this period is reported. The results in three independent experiments differed from the values reported by no more than 20% of those values.

[d] ND, not determined.

sion. A small number of syncytia were observed in clone 4 expressing the HXBc2 envelope glycoproteins. Neither of the Jurkat clones expressing the ADA envelope glycoproteins exhibited syncytium formation (Fig. 3B and data not shown).

Between 5 and 12 days after the induction of envelope glycoprotein expression, single-cell lysis was evident in the Jurkat clones expressing the 89.6 and the HXBc2 envelope glycoproteins. To focus on single-cell lysis, syncytium formation was inhibited by the addition of soluble CD4 to the Jurkat cultures. Figure 3C illustrates the results of a pilot experiment in which the Jurkat ΔKS clone or the Jurkat clones expressing the HXBc2 envelope glycoproteins were cultured in the absence of tetracycline, in either the absence or presence of soluble CD4. In the absence of soluble CD4, syncytium formation in the Jurkat clone 6 cells expressing the HXBc2 envelope glycoproteins contributed to the lower viability of these cultures compared with that of clone 4 cells. Clone 6 and clone 4 exhibited comparable levels of single-cell lysis, regardless of the presence of soluble CD4, which completely inhibited syncytium formation. Following induction, no single-cell lysis was evident in the Jurkat clone containing the ΔKS plasmid, despite the observation that levels of Rev protein comparable to those seen in the HXBc2 envelope glycoprotein-expressing clones were produced (data not shown).

To assess single-cell lysis in the Jurkat clones expressing the different HIV-1 envelope glycoproteins, the cells were cultured in the absence of tetracycline, and soluble CD4 was added to cultures that had formed syncytia in the absence of soluble CD4. The control clone containing the ΔKS plasmid and the clones expressing the ADA envelope glycoproteins grew comparably and displayed similar numbers of viable single cells in the absence or presence of tetracycline. Compared with those in these cultures, the numbers of viable cells in the Jurkat cultures expressing the 89.6 and HXBc2 envelope glycoproteins were significantly lower by 6 to 12 days following induction (Fig. 3D). In multiple experiments, 25 to 35% of the single cells in the latter cultures were trypan blue positive by 12 days after induction.

The expression of CD4 and the HIV-1 envelope glycoproteins was examined by precipitation of labeled lysates derived from cells surviving more than 2 weeks after induction. The surviving cells from the Jurkat clones expressing the HXBc2 envelope glycoproteins exhibited decreases in total CD4 expression compared with that of the uninduced cells (data not shown). In Jurkat clone 6, a decrease in the efficiency of processing of the gp160 envelope glycoprotein precursor was observed in the surviving cells compared with that seen on day 3 after induction (data not shown).

To examine the relationship between the level of envelope glycoprotein expression and single-cell lysis, clone 4 expressing the HXBc2 envelope glycoproteins and clone 2 expressing the ADA envelope glycoproteins were incubated in various concentrations of tetracycline. Aliquots of these cultures were used to assess cell viability and the level of envelope glycoprotein expression. The results are shown in Fig. 4. The percentage of single cells exhibiting lytic changes was directly related to the level of HXBc2 envelope glycoprotein expression. Even at 25% of the optimum level of HXBc2 glycoprotein expression observed in the complete absence of tetracycline, single-cell lysis in the clone 4 culture above background levels was detectable (Fig. 4D). No significant single-cell lysis was detected in the clone expressing even the highest levels of the ADA envelope glycoproteins. We conclude that while expression of the HXBc2 and 89.6 envelope glycoproteins leads to single-cell lysis in Jurkat lymphocytes, expression of the ADA envelope glycoproteins does not mediate any apparent detrimental effects on Jurkat lymphocyte growth or viability. This result suggests that specific properties of the envelope glycoproteins other than the level of expression are required for the induction of single-cell lysis.

Inducible expression of HIV-1 envelope glycoprotein mutants in Jurkat lymphocytes. To test whether specific HIV-1 envelope glycoprotein functions are required for single-cell lysis, HXBc2 envelope glycoprotein mutants were inducibly expressed in Jurkat lymphocytes. Mutant envelope glycoproteins containing the fewest amino acid changes required to effect at least a 90% reduction in a specific envelope glycoprotein function were selected from a previously characterized panel. The change of D-368 to R (368 D/R change) in the gp120 envelope glycoprotein results in at least a 100-fold reduction in CD4 binding affinity but does not affect precursor processing, gp120-gp41 subunit association, or transport to the cell surface (63). The 508/511 RR/SS changes near the gp120 carboxyl terminus result in an approximately 10-fold reduction in proteolytic processing of the gp160 precursor, with retention of CD4-binding ability and cell surface expression (5). The 522 F/Y change in the gp41 amino-terminal "fusion peptide" does not affect precursor processing, subunit association, CD4 binding, or cell surface transport but results in at least a 20-fold reduction in the ability to fuse cell membranes in a syncytium formation assay or to support virus entry (4). The 713 STOP mutant expresses an HXBc2 envelope glycoprotein with a complete truncation of the gp41 cytoplasmic tail. Peptides corresponding in sequence to portions of the HIV-1 gp41 cytoplasmic tail have been shown to bind calmodulin and to disrupt cellular membranes, both of which have been proposed to play a role in the cytopathic effects of HIV-1 and HIV-2 infection (10, 59–61, 72). An additional mutant containing both the 522 F/Y and 713 STOP changes (522+713) was constructed and tested.

The expression of the HXBc2 envelope glycoprotein mu-

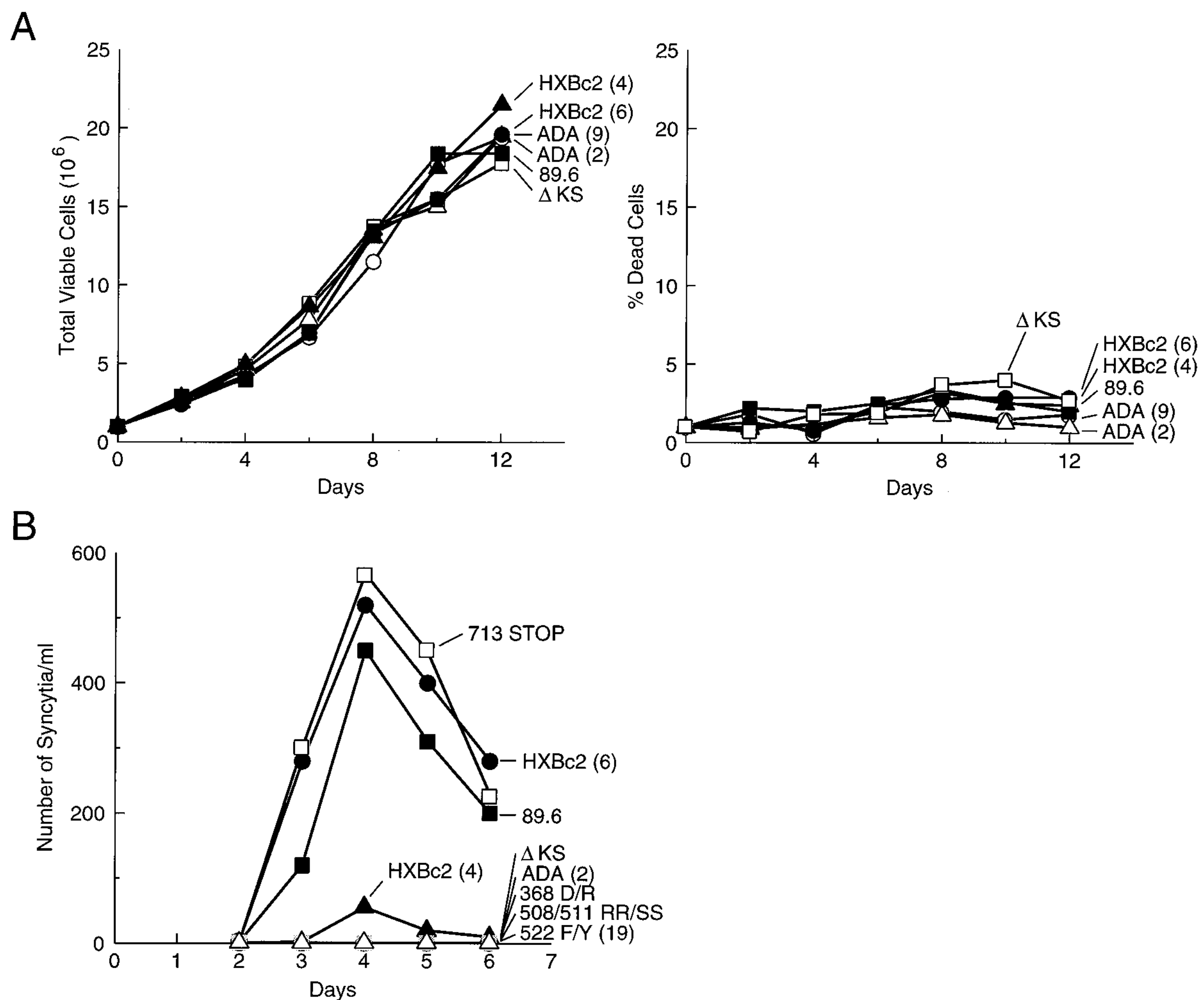

FIG. 3. Growth and viability of Jurkat clones expressing the 89.6, ADA, and HXBc2 envelope glycoproteins. (A) The total numbers of viable cells and the percentages of single cells that incorporate trypan blue (dead cells) are shown for the Jurkat clones grown in the presence of 1 μg of tetracycline per ml. (B) Number of syncytia observed following induction of expression of different envelope glycoproteins. (C) Total numbers of viable cells and percentages of dead cells associated with HXBc2 envelope expression in Jurkat cells cultured in the absence or presence of 10 μg of soluble CD4 (sCD4) per ml. (D) Total numbers of viable cells and percentages of dead cells for Jurkat clones expressing different envelope glycoproteins. To inhibit syncytium formation, soluble CD4 (10 μg/ml) was added to the Jurkat clone expressing the 89.6 glycoproteins and to clone 6 expressing the HXBc2 glycoproteins.

tants in selected Jurkat clones is shown in Fig. 2. With the exception of the clones expressing envelope glycoproteins with truncations of the gp41 cytoplasmic tail (713 STOP and 522+713), comparable levels of mutant envelope glycoprotein expression were achieved in these Jurkat clones. Truncation of the HIV-1 gp41 cytoplasmic tail has previously been observed to decrease steady-state levels of envelope glycoprotein expression (27), and despite the examination of many Jurkat clones, none expressing levels of the truncated proteins higher than those shown in Fig. 2 were identified. Consistent with previous studies (4, 63), the processing and subunit association of the 368 D/R and 522 F/Y mutants were comparable to those of the wild-type HXBc2 envelope glycoproteins. As expected, the processing of the 508/511 RR/SS precursor glycoprotein was significantly reduced, although not completely eliminated, compared with that of the wild-type HXBc2 envelope glycoprotein.

The expression of the CD4 glycoprotein on the surface of Jurkat clones containing the wild-type and mutant *env* genes, in the presence and absence of tetracycline, is shown in Table 1. In the presence of tetracycline, the surface CD4 expression of all of the Jurkat clones fell between those of the two wild-type HXBc2 clones (clones 4 and 6). Induction of envelope glycoprotein expression by culturing the cells in the absence of tetracycline resulted in a decrease in cell surface CD4 expression for all of the clones examined, except for the clone producing the 368 D/R mutant. This was expected, since the lower binding affinity for CD4 exhibited by this mutant decreases the efficiency of intracellular complexing between the envelope glycoproteins and CD4, which is the basis for the observed down-regulation of cell surface CD4 (36, 39, 74).

The effects of expression of the mutant and wild-type HXBc2 envelope glycoproteins on the growth and viability of the Jurkat clones are shown in Fig. 5 and Table 1. In the presence of tetracycline, all of the clones grew equivalently, comparably to the control clone containing the ΔKS construct (Fig. 5A). In the absence of tetracycline, syncytium formation was observed in Jurkat cells expressing the 713 STOP mutant, at a level even greater than that observed for clone 6 expressing the wild-type HXBc2 envelope glycoproteins (Fig. 3B and Table 1). No syncytia were observed in the Jurkat clones expressing the other mutant glycoproteins.

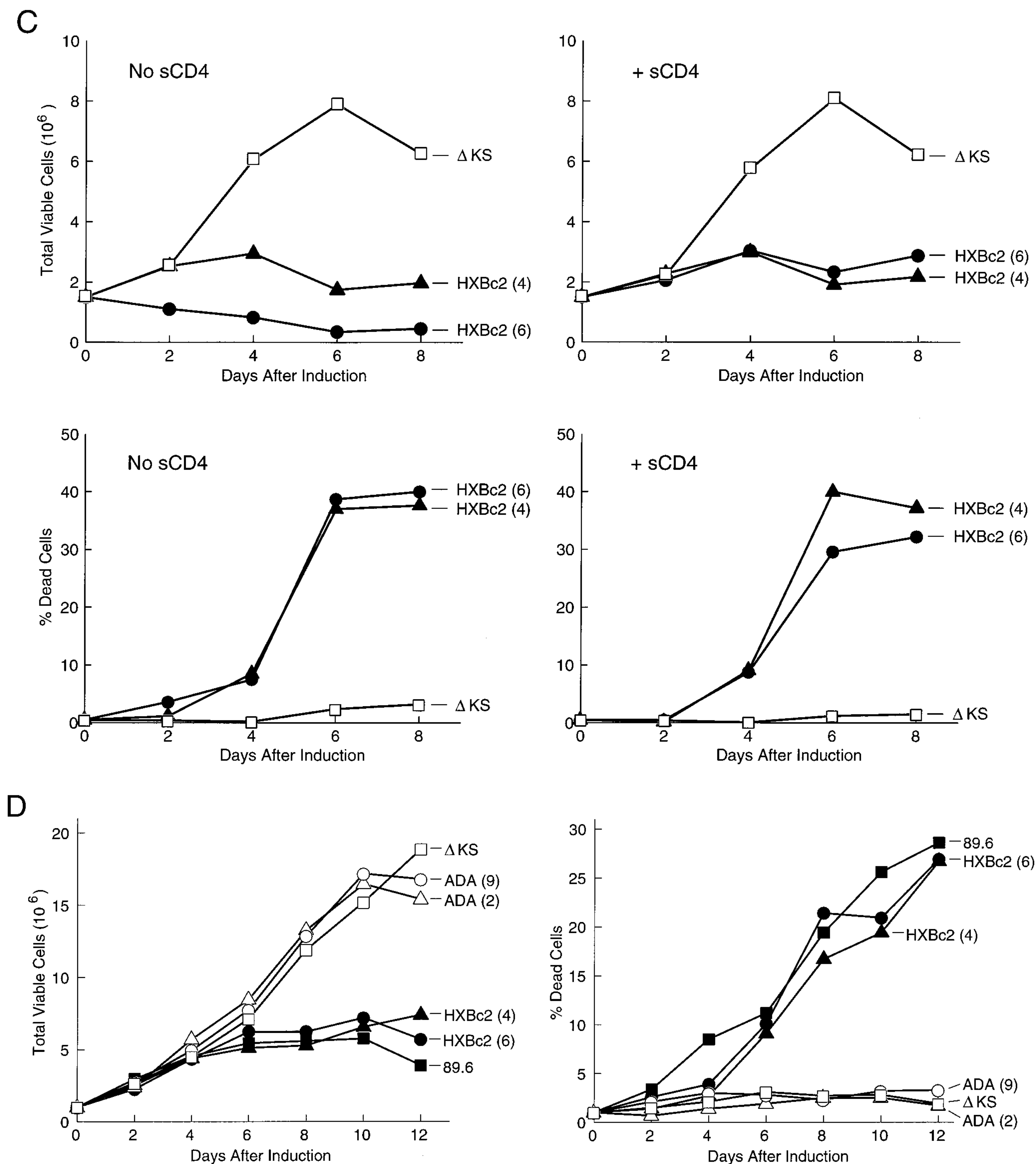

FIG. 3—*Continued.*

The growth and viability of the Jurkat clones expressing the mutant and wild-type HXBc2 envelope glycoproteins in the absence of tetracycline are shown in Fig. 5B and C. In these experiments, soluble CD4 was added to Jurkat clone 6 expressing the wild-type HXBc2 envelope glycoproteins and to the cells expressing the 713 STOP mutant. The addition of soluble CD4 completely inhibited the formation of syncytia in these cultures so that the effects of envelope glycoprotein expression on single-cell lysis could be examined. The 368 D/R change reduced but did not completely eliminate single-cell lysis. The 508/511 RR/SS mutant was dramatically attenuated in the ability to lyse single cells, although at later time points after in-

duction, a level of single-cell lysis greater than that seen in the ΔKS control was observed. The growth and viability of the two clones expressing the 522 F/Y mutant glycoproteins did not differ from those of the ΔKS control clone. The Jurkat clone expressing the 713 STOP mutant exhibited levels of single-cell lysis at least as great as those of the clones expressing the wild-type HXBc2 glycoproteins. Figure 5C shows that the Jurkat clones expressing the envelope glycoproteins with both the 522 F/Y and 713 STOP changes exhibited significantly less single-cell lysis than that observed for the 713 STOP mutant. Single-cell lysis associated with expression of the 522+713 mutant in one of the Jurkat clones was slightly greater than that seen in the clones expressing the 522 F/Y mutant glycoproteins. These results indicate that decreases in CD4 binding affinity, membrane fusion ability, or precursor processing can decrease the efficiency of single-cell lysis, whereas deletion of

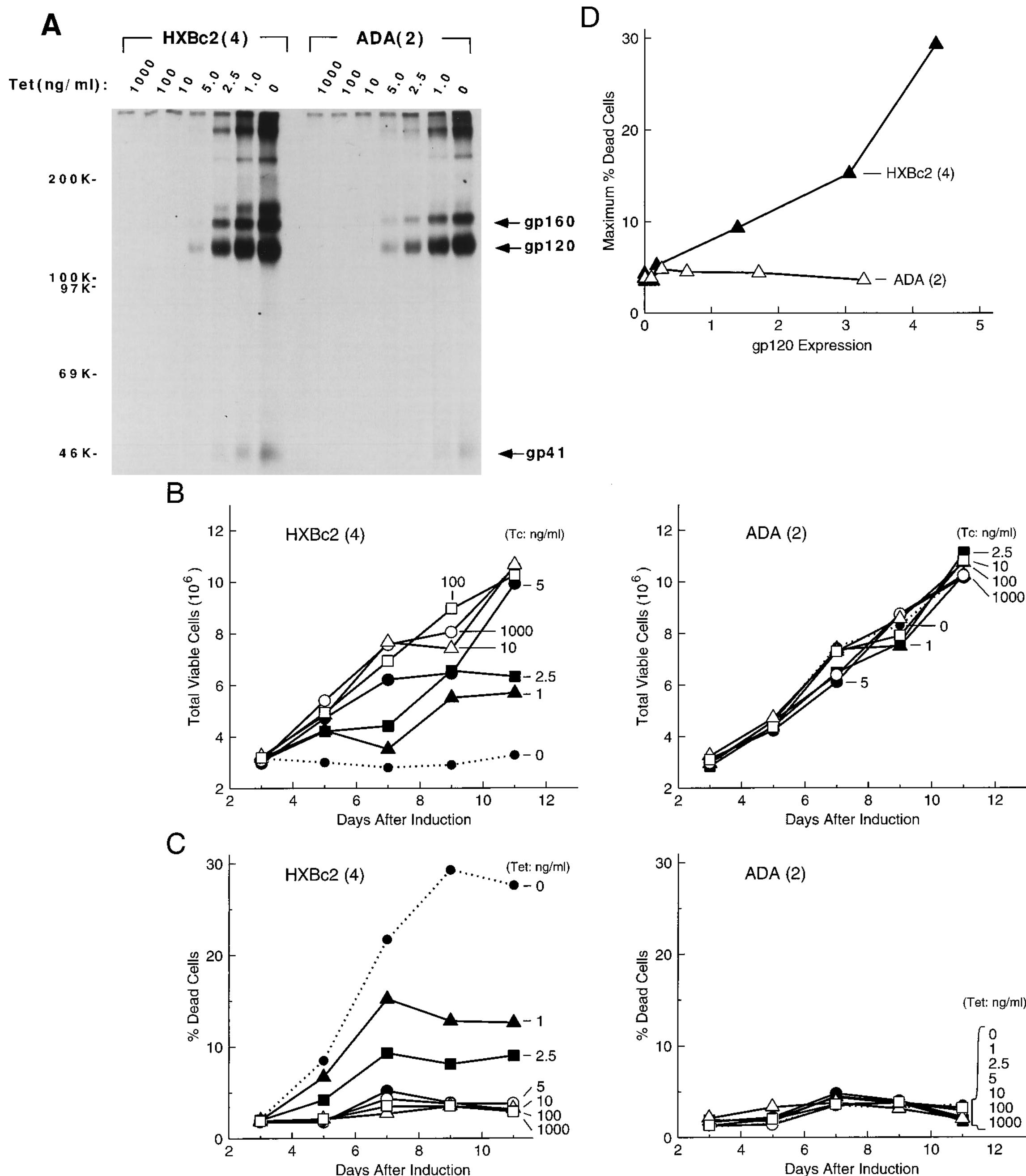

FIG. 4. Envelope glycoprotein expression level and single-cell lysis. (A) The HXBc2 envelope glycoproteins expressed in Jurkat clone 4 and the ADA envelope glycoproteins expressed in Jurkat clone 2 were precipitated with sera from HIV-1-infected individuals. The Jurkat clones were incubated and labeled in the presence of the indicated concentrations of tetracycline (Tet). (B) Total numbers of viable cells in the Jurkat cultures expressing the HXBc2 and ADA envelope glycoproteins. The concentrations of tetracycline (Tc) in the medium are indicated. (C) Percentages of single cells that incorporate trypan blue (dead cells) for the Jurkat clones expressing the HXBc2 and ADA envelope glycoproteins. The concentrations of tetracycline (Tet) in the medium are shown. (D) Maximum percentages of trypan blue-positive cells observed in the Jurkat cultures expressing the indicated levels of HXBc2 and ADA envelope glycoproteins. The level of envelope glycoprotein expression is denoted in arbitrary densitometric units.

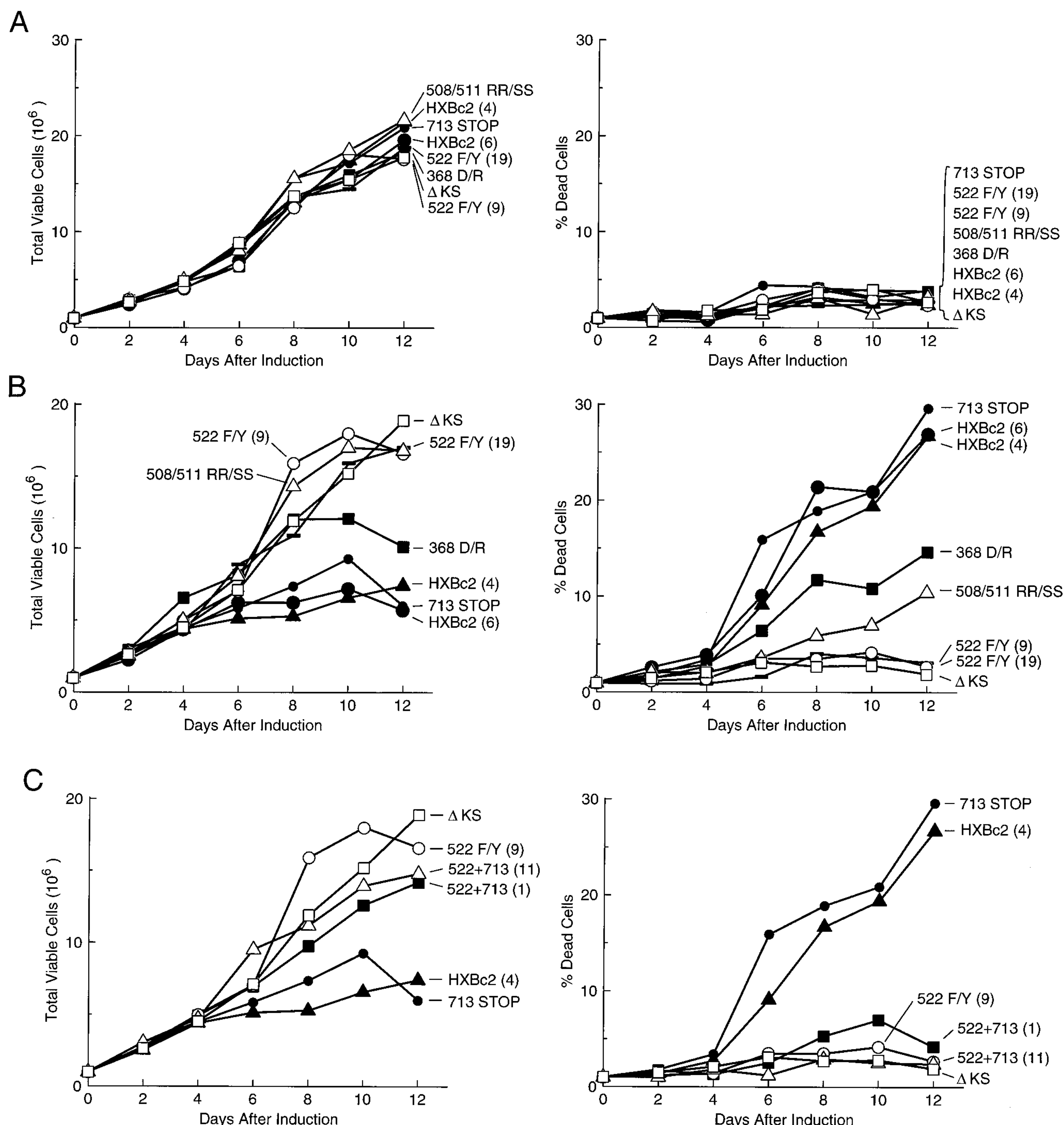

FIG. 5. Growth and viability of Jurkat clones expressing the wild-type and mutant HXBc2 envelope glycoproteins. (A) Total numbers of viable cells and percentages of dead cells in Jurkat clones cultured in the presence of 1 μg of tetracycline per ml. (B and C) Total numbers of viable cells and percentages of dead cells in Jurkat clones following induction of wild-type and mutant HXBc2 envelope glycoprotein expression. Soluble CD4 (10 μg/ml) was added to the Jurkat clone expressing the 713 STOP mutant and to Jurkat clone 6 expressing the wild-type HXBc2 envelope glycoproteins. Results similar to those shown were obtained with another Jurkat clone expressing the 508/511 RR/SS mutant.

the gp41 cytoplasmic tail does not attenuate, and may even increase the efficiency of, single-cell lysis. Furthermore, since expression of the 522 F/Y envelope glycoproteins resulted in no detectable detrimental effects on cell growth or viability, CD4 binding and the presence of the gp41 cytoplasmic tail are not sufficient to mediate toxic effects in Jurkat lymphocytes.

Characterization of Jurkat cells undergoing lysis. To determine whether single-cell lysis involved necrosis or apoptosis, Jurkat clone 4 expressing the wild-type HXBc2 envelope glycoproteins was examined by a number of techniques. In some of these experiments, Jurkat clone 2 expressing the ADA envelope glycoproteins was included as a negative control.

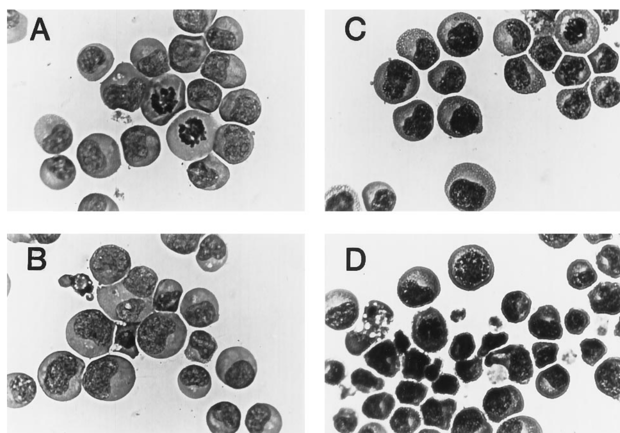

FIG. 6. Wright-Giemsa staining of Jurkat cells expressing the ADA and HXBc2 envelope glycoproteins. The Jurkat ADA(2) (A and B) and HXBc2(4) (C and D) clones were cultured either in the presence of 1 μg of tetracycline per ml (A and C) or for 9 days in the absence of tetracycline (B and D).

Figure 6 shows the morphologic appearance of Wright-Giemsa-stained HXBc2(4) and ADA(2) Jurkat clones in the presence and absence of tetracycline. Cells with apoptotic changes constituted less than 3% of the cells in the cultures grown in the presence of tetracycline (Fig. 7). When the ADA(2) clone was cultured for 12 days in the absence of tetracycline, no significant increase in the percentage of apoptotic cells was seen. Although a twofold increase in the percentage of apoptotic cells in the HXBc2(4) clone was seen following culture in the absence of tetracycline, a significantly greater increase (14- to 15-fold) in the percentage of trypan blue-positive cells in the culture was evident. This indicates that the majority of the Jurkat cells dying as a result of the expression of the HXBc2 envelope glycoproteins do not demonstrate changes typical of apoptotic cells.

Examination of the HXBc2(4) Jurkat clone cultured in the absence of tetracycline by electron microscopy confirmed that the majority of the single cells exhibiting cytopathology did not demonstrate apoptotic features (Fig. 8). Mitochondrial swelling and loss of mitochondrial cristae, which are features of necrotic cells (50, 55, 85), were observed in the HXBc2(4) cells by 6 days following induction of envelope glycoprotein expression (Fig. 9).

Figure 10 shows that very few apoptotic cells were detected by TUNEL in the HXBc2(4) or ADA(2) clones that were expressing the HIV-1 envelope glycoproteins. Control staurosporine-treated cells (38) demonstrated significant labeling by this method. Consistent with these results, internucleosomal fragmentation of cellular DNA was observed for staurosporine-treated cultures but not for Jurkat cells undergoing lysis as a result of HIV-1 envelope glycoprotein expression (data not shown).

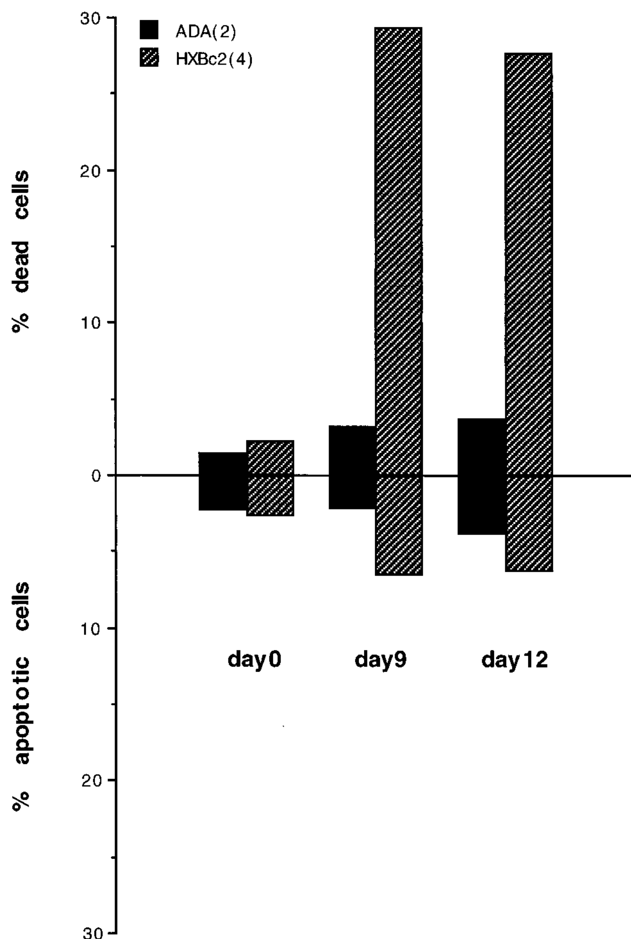

FIG. 7. Measurement of apoptotic cells in Jurkat cultures expressing the HXBc2 and ADA envelope glycoproteins. Giemsa staining was used to estimate the percentage of apoptotic cells in clone 2 expressing the ADA envelope glycoproteins and in clone 4 expressing the HXBc2 envelope glycoproteins at various times after induction. At these times, the percentage of single cells incorporating trypan blue was also estimated.

DISCUSSION

Our results demonstrate that both of the acute cytopathic effects associated with HIV-1 infection, syncytium formation and single-cell lysis, involve membrane fusion mediated by the viral envelope glycoproteins. In the inducible system used for envelope glycoprotein expression, the appearance of syncytia was invariably followed by single-cell lysis. It has been previously observed that some target cells for HIV-1 infection exhibit single-cell lysis but few or no syncytia and that some changes in the HIV-1 envelope glycoproteins attenuate syncytium formation more than the ability of the virus to lyse single cells (6, 17, 47). The requirement for a greater number of successful envelope glycoprotein-CD4 interactions for syncytium formation than for single-cell lysis could explain these observations. Since, in our experimental system and in HIV-1-infected cells, the level of envelope glycoprotein expression usually exceeds that of CD4, the appearance of particular cytopathic effects is more often limited by the latter parameter. For example, even though clone 4 expresses slightly higher levels of the wild-type HXBc2 envelope glycoproteins than does clone 6, the low levels of CD4 expressed in clone 4 might account for the observed lack of syncytia. These two clones exhibited comparable levels of single-cell lysis, consistent with a lower number of envelope glycoprotein-receptor interactions being sufficient for this form of cytopathic effect than for the formation of syncytia.

A comparison of the single-cell lyses accompanying the expression of the wild-type and mutant HXBc2 envelope glycoproteins provides important clues to the mechanisms underlying the resultant cell damage. Because priority was given to the selection of mutants that exhibited discrete defects in specific envelope glycoprotein functions, the phenotypes of the mutants are not absolute. For example, by using sensitive assays,

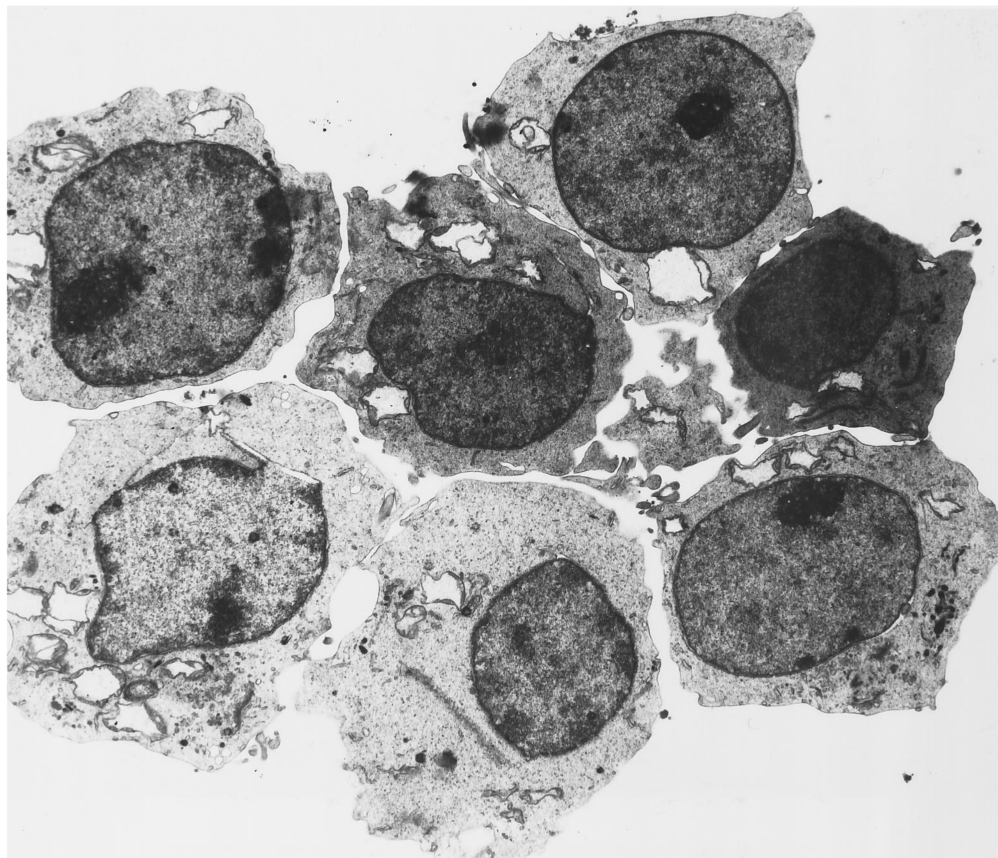

FIG. 8. Low-magnification electron micrograph of Jurkat clone 4 expressing the HXBc2 envelope glycoproteins, 6 days after propagation of the cells in medium lacking tetracycline.

some residual receptor-binding and membrane-fusing activity could be detected for the 368 D/R and 522 F/Y mutants, respectively (4, 63). Even the 508/511 RR/SS mutant, containing two amino acid changes at the gp120-gp41 cleavage site, exhibited a small amount of residual precursor processing. Nonetheless, the data indicate the importance of gp160 processing, CD4 binding, and membrane fusion for efficient single-cell lysis. The 508/511 RR/SS mutant was significantly impaired in single-cell lysis ability compared with the wild-type HXBc2 envelope glycoproteins. The low level of single-cell lysis detectable greater than 9 days after the induction of 508/511 RR/SS expression can be attributed to the residual precursor processing that occurs despite the amino acid changes at the cleavage site.

The 368 D/R mutant, which is deficient in CD4 binding (63), is completely defective in syncytium formation. This mutant exhibited a significant although not absolute attenuation in single-cell lysis ability. The basis for this residual activity is not clear. One possibility is that other mechanisms besides CD4 binding can trigger single-cell lysis events, but previous studies demonstrated that the expression of functional HIV-1 envelope glycoproteins can be tolerated without cytopathic effects in CD4-negative cells (44). A more likely explanation is that high local concentrations of CD4 in the intracellular compartments relevant to single-cell lysis can partially compensate for the lower binding affinity of the 368 D/R envelope glycoproteins. Indeed, precipitation of labeled cell lysates with the OKT4 anti-CD4 antibody revealed that, although present at a lower level than that seen for Jurkat clone 6 expressing the wild-type HXBc2 envelope glycoproteins, complexes of CD4 and the 368 D/R envelope glycoproteins could be detected (data not shown).

The conservative change of phenylalanine 522, located in the gp41 amino-terminal fusion peptide (4, 26, 47), to tyrosine dramatically attenuated the lysis of single Jurkat lymphocytes. This result was seen for both the complete HXBc2 envelope glycoproteins and for envelope glycoproteins in which the gp41 cytoplasmic tail was deleted. These results demonstrate the importance of the fusion peptide to single-cell lysis and suggest that membrane fusion events are necessary for this form of cytopathic effect. Since gp160 processing and CD4 binding are prerequisites for efficient membrane fusion (5, 15, 32, 52, 56, 69), these results are consistent with those obtained for the 508/511 RR/SS and 368 D/R mutants, respectively. Since the affinity of the HIV-1 envelope glycoproteins for CD4 is not affected by the 522 F/Y change (4), the results also indicate that receptor binding is not sufficient for the induction of single-cell lysis. A necessary role for the process of membrane fusion in the lysis of HIV-1-infected cell lines and primary lymphocytes explains the attenuated cytopathic effects observed for replication-competent viruses with fusion-altering changes in the envelope glycoproteins (6, 47, 73).

Peptides corresponding in sequence to portions of the HIV-1 gp41 cytoplasmic tail have been shown to bind calmodulin and to lyse cell membranes (10, 59–61, 72). These observations have led to the speculation that the gp41 cytoplasmic tail may exert detrimental effects on the viability of HIV-1-infected cells. Such effects were not observed in this study. For

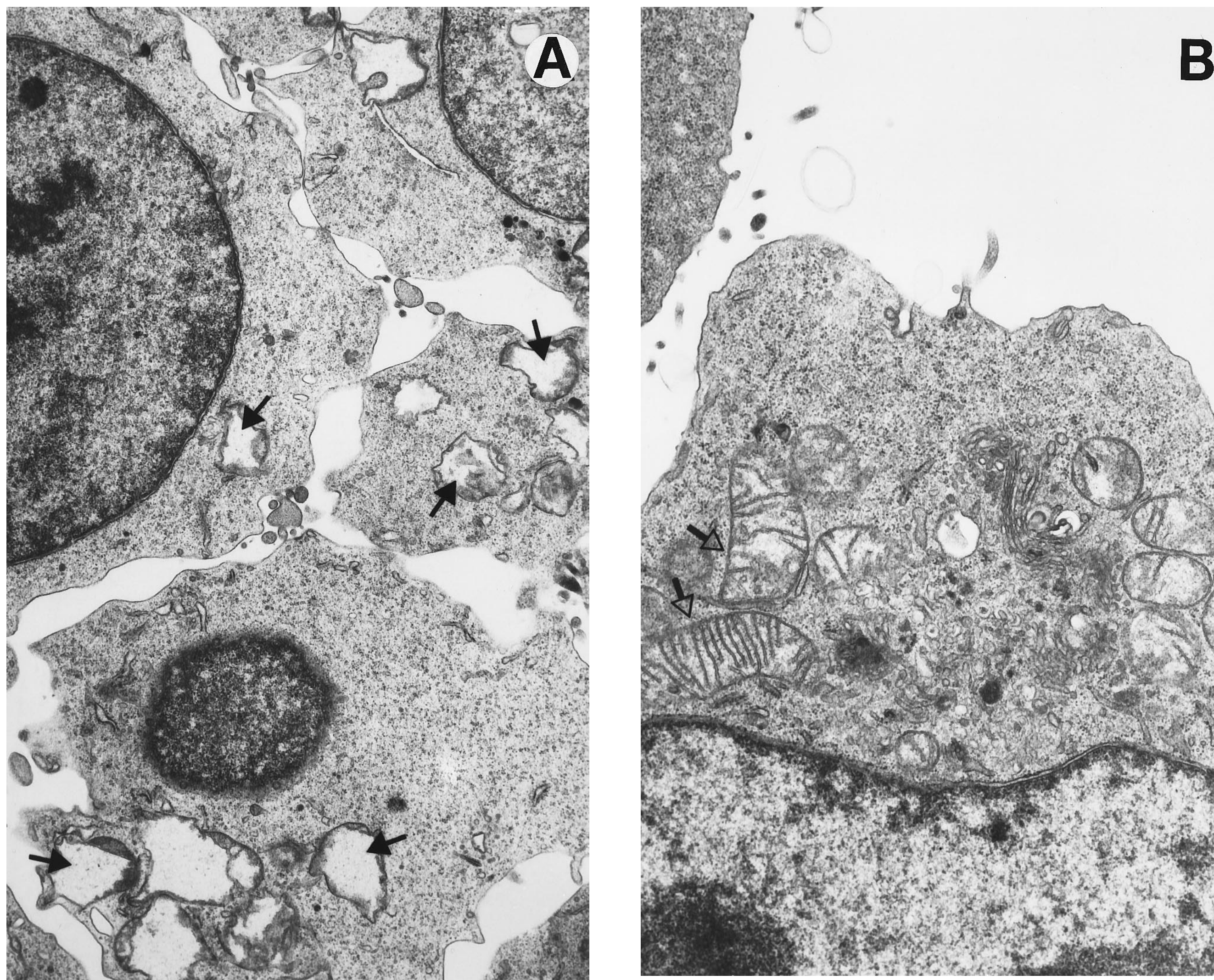

FIG. 9. High-magnification electron micrographs of Jurkat clone 4 expressing the HXBc2 envelope glycoproteins. (A) HXBc2(4) clone after 6 days of culture in medium lacking tetracycline. The arrows indicate swollen mitochondria with loss of cristae. (B) HXBc2(4) clone cultured in the presence of 1 μg of tetracycline per ml. The arrows indicate mitochondria.

example, the growth and viability of Jurkat lymphocytes expressing the ADA or HXBc2 522 F/Y envelope glycoproteins were indistinguishable from those of the ΔKS control cells expressing only the Rev protein. While the HIV-1 gp41 cytoplasmic tail may exert effects on the viability of cells other than those examined here, the expression of this domain is not sufficient to induce cytolysis or to influence the growth of Jurkat lymphocytes.

The deletion of the HIV-1 gp41 cytoplasmic tail resulted in a significant decrease in the steady-state levels of expression of the mutant envelope glycoproteins, consistent with a previous report (27). Despite this, the formation of syncytia and the lysis of single cells by these mutants were at least as efficient as those observed for the full-length envelope glycoproteins. In one of two Jurkat clones, deletion of the gp41 cytoplasmic tail allowed a low level of single-cell lysis to be detected for the 522 F/Y mutant. These results are consistent with those of previous studies indicating that the fusogenicity of the HIV-1 and HIV-2 envelope glycoproteins, measured by syncytium-forming ability, can be enhanced by truncation of the gp41 cytoplasmic tail (27, 48, 61). The retention of single-cell lysis ability by the tail-deleted mutants in our study is also consistent with reports indicating that HIV-1 viruses lacking portions of the gp41 cytoplasmic tail are still cytopathic (78, 81).

How might membrane fusion events mediated by the envelope glycoproteins lead to the death of single cells? As was seen in HIV-1-infected cultures (3), single-cell lysis in our system was not inhibited by soluble CD4, and at the time of maximal single-cell lysis, the surface expression of the CD4 glycoprotein was low in many cases. These observations suggest that the HIV-1 envelope glycoprotein–CD4 interactions that initiate membrane fusion events relevant to single-cell lysis occur intracellularly. Since gp160 processing, which is essential for activating the fusogenic potential of the envelope glycoproteins (56), occurs in the Golgi apparatus (20, 21, 82), this organelle represents a likely site in which membrane damage is initiated. As Golgi vesicles are normally fused to the plasma membrane during exocytosis, the integrity of the plasma membrane may also be compromised. HIV-1-infected cells have been reported to exhibit increased membrane per-

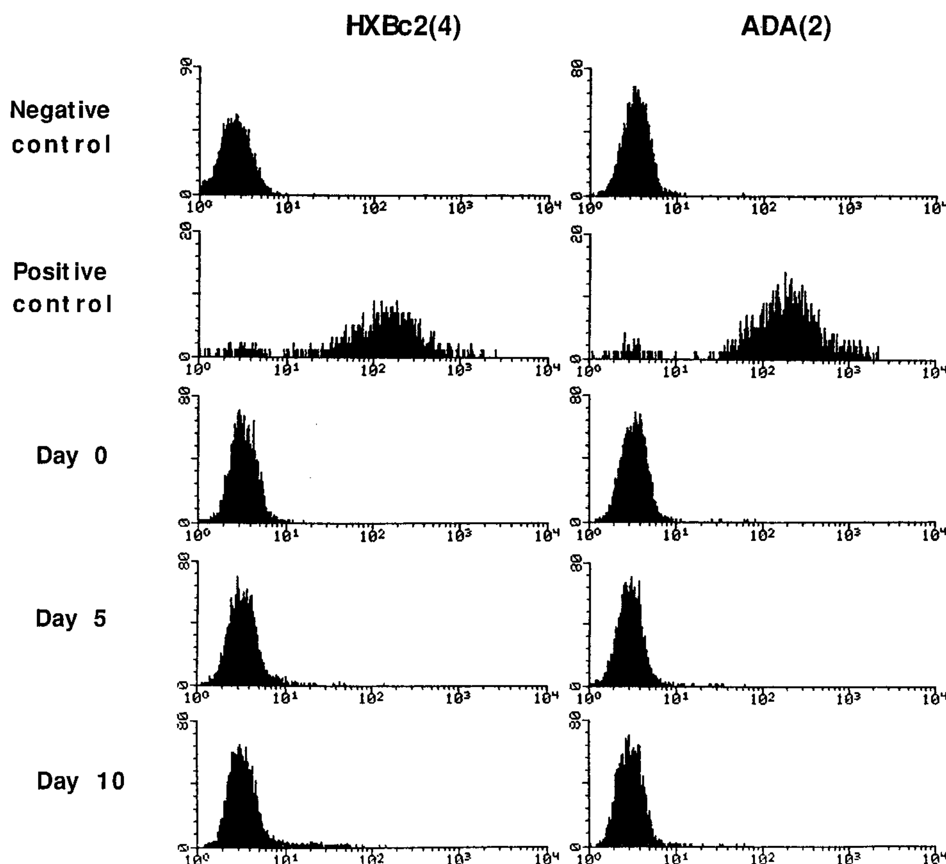

FIG. 10. TUNEL analysis of Jurkat clones expressing the HXBc2 and ADA envelope glycoproteins. The HXBc2(4) and ADA(2) Jurkat clones were cultured in the presence of tetracycline (1 μg/ml) plus PBS (negative control), tetracycline (1 μg/ml) plus staurosporine (positive control), or tetracycline (1 μg/ml) in medium (day 0) or in the absence of tetracycline for 5 or 10 days (day 5 and day 10). The cells were subjected to TUNEL analysis as described in Materials and Methods.

meability (12, 54). As was seen in HIV-1-infected Jurkat cells (3, 6, 47), the time course of single-cell lysis is later than that of syncytium formation in our system. HIV-1 infection of the Jurkat clones grown in the presence of tetracycline resulted in the same time course of cytopathic effects (data not shown). This temporal pattern suggests that single-cell lysis may be the result of cumulative sublethal insults. The continuous membrane-disrupting consequences of envelope glycoprotein-CD4 interactions may eventually overwhelm the compensatory and repair capabilities of the cell.

Most of the Jurkat lymphocytes exhibiting decreased viability, as judged by the inability to exclude trypan blue, demonstrated changes characteristic of necrosis. The minority of lymphocytes undergoing single-cell lysis exhibited apoptotic features. This situation is similar to that seen in HIV-1-infected cultures (65) and suggested that apoptosis is not a necessary path to cell death induced by HIV-1.

Koga and colleagues have used a metallothionein promoter, which is inducible with zinc or cadmium, to study the effects of HIV-1 envelope glycoprotein expression on Jurkat lymphocytes and on U937 monocytes (41–44). In light of the studies presented here, there are several puzzling aspects of their results. First, upon addition of metals, their envelope glycoprotein-expressing cells were lysed within 3 days, with a significant fraction of the cells demonstrating apoptotic changes (42, 44, 53). Both the timing and the degree of apoptosis observed were different from those seen for HIV-1-infected Jurkat cells undergoing single-cell lysis (3, 6, 47, 65). One possible explanation is that the metals used for the induction of envelope glycoprotein expression contributed to the toxic phenotype. An advantage of the tetracycline-inducible system used in the present work is that induction involves the removal of an agent from, rather than the addition of an agent to, the culture. Second, an envelope glycoprotein mutant containing a single amino acid change (equivalent to 511 R/S in our numbering system) at the gp160 cleavage site was reported to induce efficient single-cell lysis (41). On the basis of this observation, Koga et al. concluded that processing of the envelope glycoprotein precursor was not necessary for single-cell killing (41). This conclusion is incompatible with the observation that replication-competent HIV-1 viruses containing envelope glycoproteins with partial defects in either proteolytic processing or membrane fusion exhibited decreases in single-cell lysis (6, 47, 73). Their result also contrasts with that obtained for the 508/511 RR/SS mutant in our study and with the observation that selection of clones exhibiting inefficient gp160 processing occurs in cell lines stably expressing high levels of CD4 and HIV-1 envelope glycoproteins (76). One explanation for these discrepancies is that the mutant used by Koga and colleagues is not completely defective in precursor processing, a possibility suggested by our observation that even changes in two amino acids near the cleavage site did not absolutely eliminate gp160 processing. Indeed, inspection of the report of Koga et al. (41) indicates that a significant amount of processed gp120 glycoprotein is evident in the induced U937 cells. On the basis of results generated by using their system, Koga and colleagues

have suggested that single-cell lysis in HIV-1 infection results from a blockade of cellular transport through nuclear pores by envelope glycoprotein-CD4 complexes (42, 43). Our results, as well as those obtained by the study of cells infected with HIV-1 mutants (6, 47, 73), suggest that the binding of the envelope glycoproteins to CD4 is insufficient to cause cell lysis and render such a model unlikely. It is possible that nuclear transport is affected as a secondary consequence of envelope glycoprotein-mediated membrane disruption (31) rather than representing a primary event in cell killing.

Envelope glycoproteins derived from both laboratory-adapted (HXBc2) and primary clinical (89.6) HIV-1 isolates were able to mediate single-cell lysis in Jurkat lymphocytes. Jurkat lymphocytes possess the incompletely characterized cellular factors required for efficient membrane fusion by the HXBc2 and 89.6 envelope glycoproteins, as evidenced by the high level of syncytium formation that accompanied expression of these proteins. By contrast, even though the envelope glycoproteins from another primary HIV-1 isolate (ADA) were expressed at high levels and down-regulated surface CD4 expression, no single-cell lysis was observed. The inability of the ADA envelope glycoproteins to fuse Jurkat cell membranes is likely to represent one factor limiting single-cell lysis, in light of the results obtained with fusion-defective HXBc2 mutants. Recombinant HIV-1 viruses containing the ADA envelope glycoproteins have been shown to enter primary human peripheral blood mononuclear cells less efficiently than viruses containing the 89.6 or HXBc2 envelope glycoproteins (75). This decrease in the functional activity of the ADA envelope glycoproteins may account for the observation that, even when virus replication is normalized, the ADA virus is less cytopathic for peripheral blood mononuclear cells than are the 89.6 and HXBc2 viruses (86).

Besides the envelope glycoproteins, other HIV-1 proteins have been shown to exert detrimental effects on cells expressing these products. Cells overexpressing the HIV-1 protease are counterselected after several weeks in culture (49), and the Vpr protein can mediate cell cycle arrest (68). Either of these effects could influence the efficiency with which cells chronically producing infectious viruses are established. However, since most HIV-1-producing cells in vivo exhibit half-lives of only 2 to 3 days (34, 79), these chronic toxic or cytostatic effects may have little influence on the fate of the majority of infected CD4-positive lymphocytes. Further studies will be required to understand the relative contributions of the acute viral cytopathic effect, chronic detrimental effects of other viral proteins, and immune clearance to CD4 lymphocyte destruction in vivo.

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