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Rapid Induction of Apoptosis by Cell-to-Cell Transmission of Human Immunodeficiency Virus Type 1

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The kinetics of human immunodeficiency virus type 1 (HIV-1)-induced cell death were investigated in cell-to-cell and cell-free models of virus transmission. Cocultivation of HIV-1 chronically infected H9 donor cells with uninfected H9 recipient cells resulted in rapid induction of programmed cell death. Within 8 h, apoptotic chromatin condensation was identified by histologic staining. In addition, many single cells with apoptotic nuclei were observed, indicating that stable cell fusion was not a requirement for apoptosis to occur. By 12 to 18 h of coculture, a DNA fragmentation ladder characteristic of apoptosis was detected by agarose gel electrophoresis. Quantitation of apoptosis by measurement of nuclear DNA content revealed that at least 20 to 30% of the nuclei were undergoing apoptosis by 24 h after cocultivation. The appearance of condensed nuclei and fragmented DNA occurred as HIV reverse transcription was completed, and it was not inhibited by zidovudine, suggesting that induction of apoptosis did not require new HIV replication. Soluble CD4 inhibited apoptosis, demonstrating that Env-CD4 interactions were required for apoptosis. In contrast to that in cell-to-cell transmission, apoptosis in cell-free HIV infections was markedly inefficient and was not observed until 70 to 90 h after infections were initiated. These findings indicate that HIV-1 induction of programmed destruction of the nucleus is initiated at the time of cell-cell cocultivation by a mechanism which requires CD4-Env interactions but not new HIV replication.

Human immunodeficiency virus type 1 (HIV-1) is transmitted to susceptible $CD4^+$ cells by virion particles or infected cells. Although several critical steps in the HIV life cycle have been identified, the consequences of virus replication events for host cells are only poorly understood. HIV infection results in marked cytopathic effects in cultured T-lymphocytic cell lines, but the mechanisms of cell death remain unclear. Recently, several investigators (2, 3, 12, 25, 30, 32, 33, 43, 52) suggested that HIV infection initiates programmed cell death, a sequence of nuclear reorganization and condensation events (7, 40, 57) which may culminate in apoptosis, with DNA digestion into megabase-sized (40, 41) or oligonucleosome-sized fragments (7). Laurent-Crawford and coworkers (25) have reported that HIV *env* gene products gp120 and gp41 were both required to induce apoptosis by engaging the HIV-1 receptor, CD4, suggesting that the cytopathic effect of HIV may be initiated upon virus entry. In contrast, several investigators have suggested that the cytopathic effects of HIV occur as a consequence of virus replication. Cloyd and Lynn (6) reported significant alterations in the lipid composition of host membranes after HIV infection, resulting in increased membrane permeability; others (4, 24, 30, 49) have reported toxic effects of *env* expression in *env*-inducible cell lines or in acute HIV infections. Klimkait and coworkers (23) noted increased cytopathic effect in cells infected with a vpu^- HIV strain compared with that detected in cells infected with isogenic vpu^+ virus. In virus expression studies, the asynchronous character of cell-free HIV infections did not permit direct comparison of the kinetics of virus replication and the induction of cell death.

We and others have recently characterized a cell-cell model for HIV transmission which provides a rapid and synchronous method for HIV transmission (29, 47). Coculture of HIV-1 chronically infected H9 cells with uninfected H9 cells results in new HIV-1 DNA synthesis of as many as 40 HIV-1 proviral copies per cell within 4 h of cocultivation and in release of progeny virions after 24 h of cocultivation. Kinetic analyses by Dimitrov et al. (8) revealed that cell-to-cell spread is 2 to 3 orders of magnitude more efficient than transmission of HIV by cell-free virions and therefore represents the predominant mode of HIV transmission after cell-free infection by HIV has taken place. In the present study, we investigated the synchronous cell-to-cell transmission model to identify the mechanism of cell death and to determine whether cell destruction was a product of HIV replication or was initiated upon virus entry. We obtained unambiguous evidence of apoptosis within 8 hours of coculture of uninfected H9 cells with H9 cells chronically infected with HIV. Programmed cell death occurred in the absence of new HIV replication and required Env-CD4 interactions. In contrast to the induction of apoptosis in the cell-to-cell transmission model, the induction of apoptosis following cell-free HIV infections was inefficient and markedly delayed. These results suggest that induction of apoptosis is principally mediated by Env-CD4 interactions between HIV-infected and uninfected cells.

MATERIALS AND METHODS

Cells and virus. The Jurkat, H9, CEM-derived 12D7, and HIV chronically infected H9 (H9IIIB) cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, glutamine, and antibiotics (maintenance medium [47]). HeLa cells were maintained in Dulbecco modified Eagle medium with supplements identical to those of the maintenance medium.

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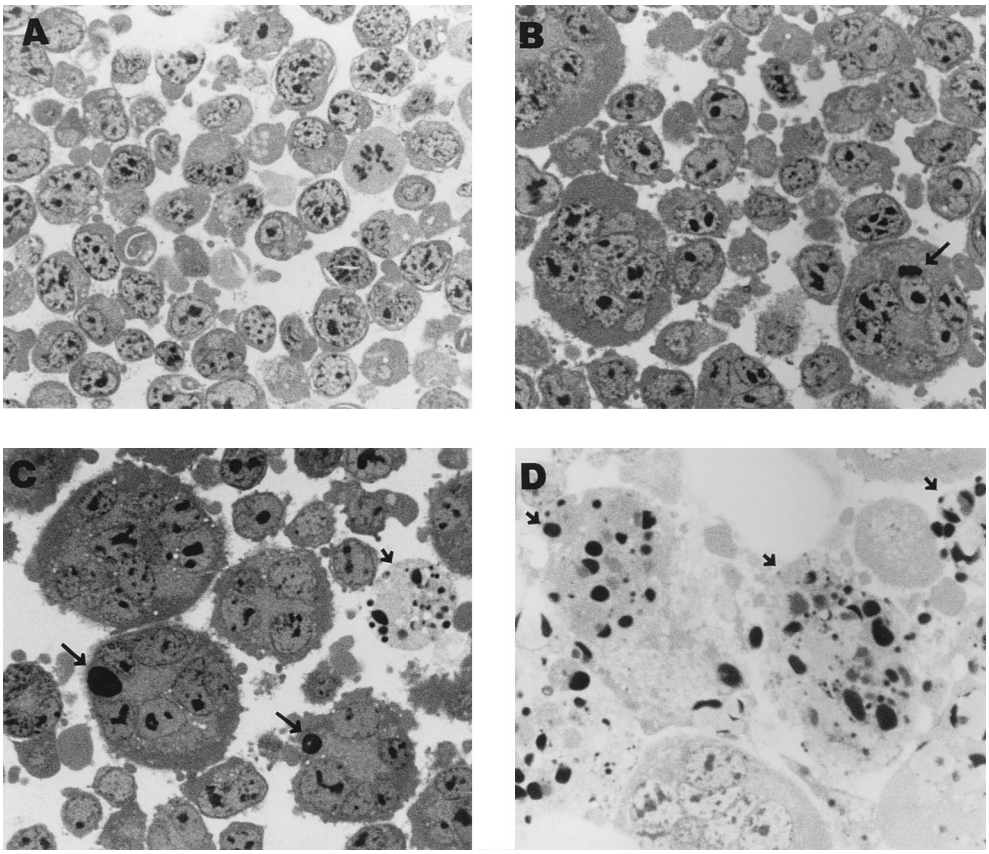


FIG. 1. Rapid induction of apoptosis in H9-H9IIIB cocultivations. H9 cells were cocultivated for 0 h (A), 2 h (B), 8 h (C), or 32 h (D) and then fixed, sectioned, and stained with methylene blue. Single apoptotic nuclei within cell fusions for 2- and 8-h cocultivations are denoted by large arrows. Nuclei in the large syncytium at 8 h and at 32 h demonstrating destruction of all nuclei within a syncytium are indicated (small arrows). Magnification, ×630.

HIV_{IIIB} was obtained from cultures of H9IIIB cells (47); culture supernatants were pelleted at 1,000 rpm for 5 min to remove cells and debris, filtered, and then pelleted at 200,000 × *g* for 1 h. The pellets were resuspended in RPMI medium and used for infections or Western blotting (immunoblotting). The infectious titer of the concentrated HIV preparation was determined by endpoint dilution in H9 cells using reverse transcriptase activity in culture supernatants to demonstrate the presence of HIV. The titer was defined as the midpoint between the last dilution positive and the first dilution negative for reverse transcriptase activity. Reverse transcriptase was measured as previously described (50).

To obtain virus stocks of HIV molecular clones, HeLa cells were transfected with 15 μg each of the HIV-1 infectious molecular clones pNL4-3 (1), pNL43U35 (50), and pALA-5 (10) by the calcium phosphate technique as described previously (50) except that the cells did not undergo glycerol shock but were washed and refed with maintenance medium. Culture medium was harvested at 48 h after transfection, clarified by centrifugation at 1,000 × *g* for 5 min to remove released cells, and filtered through 0.45-μm-pore-size filters. H9 cells chronically expressing HIV were established by infecting 2.5×10^6 cells with a multiplicity of infection of approximately 0.002 (8). After 2 to 3 weeks following the acute infection, cells chronically expressing infectious HIV without cytopathic effect grew out of the cultures and were used for cocultivation experiments.

Fluorescence-activated DNA analysis. Quantitative DNA measurements of cells were performed by using a B/D FACsort instrument with the CELLFIT DNA analysis package. DNA content of 5,000 to 10,000 cells was determined by staining of nuclei with propidium iodide as described previously (38); apoptotic nuclei were identified as a distinct hypodiploid peak in the log-scale Fl-3 channel of the cytometer and were gated and enumerated. After the analyses were performed in CELLFIT, the LYSIS II software package was used to arrange histograms.

Histologic staining. Cells were examined for apoptosis in whole mount and thin-section preparations. Whole mounts of cells attached to glass slides by cytocentrifugation or to slides treated with poly-L-lysine were stained with May-Grunwald-Giemsa (31). To clearly identify highly condensed apoptotic nuclei, the cells were destained for 2 to 5 min in water. Thin sections of cells were prepared as described previously (47) by glutaraldehyde fixation-OsO_4 postfixation; 1-μm thin sections were stained with toluidine blue. Whole mount specimens, in which entire cells were visualized, were superior to 1-μm thin sections in demonstrating hypercondensation of chromatin and fragmentation in single cells because an entire nucleus could be visualized at once.

DNA preparation and electrophoresis. To detect apoptotic DNA, unintegrated DNA was isolated by the Hirt procedure (47), digested with RNase A, and electrophoresed in 1% agarose gels at 1 mA/cm in 0.04 M Tris-acetate–0.001 M EDTA (TAE) buffer containing ethidium bromide; a 1-kb DNA marker (Bethesda Research Laboratories, Inc., Gaithersburg Md.) was used as a molecular marker. To identify HIV DNA by Southern blotting, unintegrated DNA preparations were restricted with *Msc*I and electrophoresed in 0.6% agarose gels at 3 V/cm in 1× TAE buffer; the *Hin*dIII marker (Bethesda Research Laboratories) was used as a marker. Following electrophoresis, gels were alkali fixed and DNA was transferred to nylon Hybond-N+ membranes (Amersham Life Science Products) in 0.4 N NaOH–1.5 M NaCl. Membranes were prehybridized (in 6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–1% sodium dodecyl sulfate [SDS]–10× Denhardt's solution–80 μg of sheared, boiled salmon sperm DNA per ml) and then hybridized at 42°C (in 50% formamide–6× SSC–1% SDS–7% dextran sulfate–5× Denhardt's solution–70 μg of sheared, boiled salmon sperm DNA per ml [19]) with a ^{32}P-labeled probe obtained by random hexamer priming of a mixture of *Hin*dIII fragments of the infectious molecular clone pNL4-3 which span the HIV genome from positions 536 to 9606.

Detection of HIV proteins. HIV preparations and cell extracts were prepared, electrophoresed on 10% acrylamide gels, and electroblotted onto nitrocellulose membranes as previously described (50). HIV proteins were detected by Western blotting by sequential incubation of the membranes with a 1:1,000 dilution of plasma from two HIV-seropositive persons and a 1:1,000 dilution of ^{125}I-protein A (50). HIV proteins were visualized and quantitated with the Fuji phosphorimaging system.

RESULTS

Induction of cell death by cocultivation. As previously described, cocultivation of uninfected H9 cells with H9 cells chronically infected with HIV-1 results in rapid transmission of

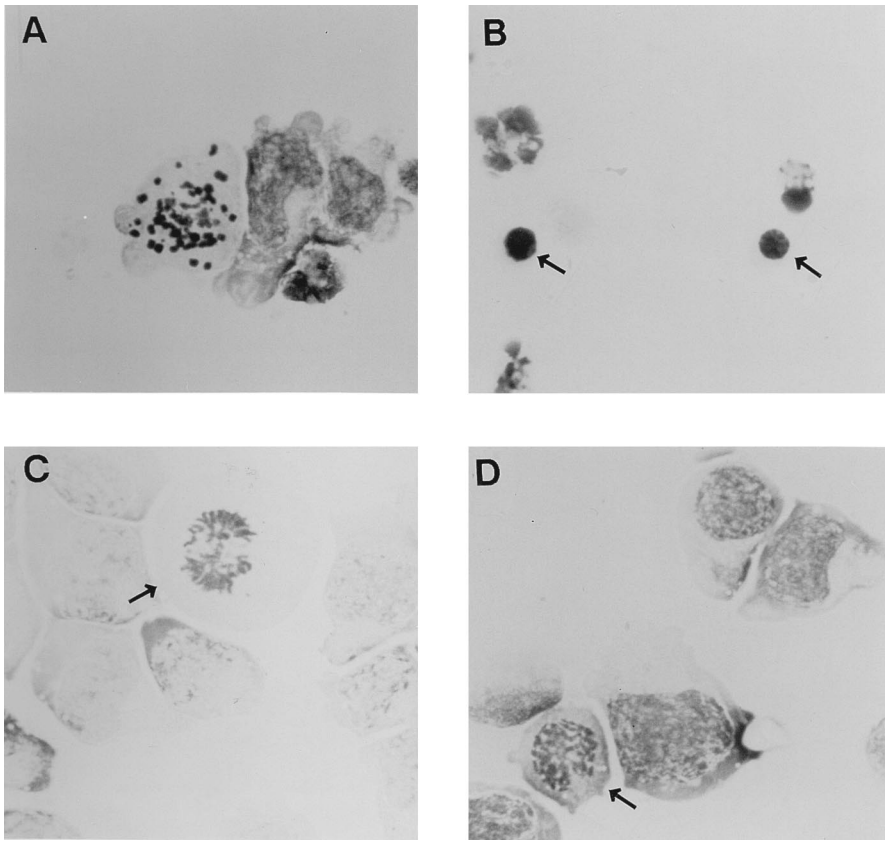


FIG. 2. Single-cell killing by apoptosis. Cocultivations were prepared by cytocentrifugation and staining with May-Grunwald-Giemsa (A and B), and apoptotic nuclei in single cells were identified by extensive fragmentation (A) and condensation (B, arrows). Control cytocentrifuged H9IIIB cells (C) and H9 cells (D) stained with May-Grunwald-Giemsa did not show any features of apoptosis. All preparations were extensively destained to demonstrate condensed nuclear fragments clearly. Magnification, ×630.

HIV to uninfected cells; new HIV DNA synthesis occurs within 4 h, and new virions are produced after 24 to 48 h (47). Previous examinations of the cocultures revealed cell-cell fusions as rapidly as 15 min following cocultivation and the appearance of large balloon-like membrane protrusions within 24 h of coculture (47). To investigate whether the cytopathic effect observed after cell-to-cell HIV transmission included programmed cell death, we analyzed the appearance of nuclei in cocultivations by light microscopy. As shown in Fig. 1, thin sections of H9-H9IIIB cocultures stained with toluidine blue demonstrated the presence of nuclei with marked condensation and fragmentation, indicating that programmed cell death had been initiated; nuclear changes were evident as early as 2 h following cocultivation, when nuclei within fused cells began to condense (compare Fig. 1B, arrow, with Fig. 1A). The frequency of nuclei with distinct condensation and fragmentation increased after HIV cell-to-cell transmission was initiated (Fig. 1B to D, arrows); by 32 h following cocultivation, most cells exhibited apoptotic changes, although some of the fused cells still contained apparently normal-appearing nuclei (Fig. 1D). In whole mount specimens, nuclei from single cells with characteristic condensation (Fig. 2B, arrow) and condensation with extensive fragmentation (Fig. 2A) were observed. Cells undergoing apoptosis were easily distinguished from typical H9IIIB cells (Fig. 2C) or H9 cells (Fig. 2D), including cells in metaphase (Fig. 2C, arrow) or prophase (Fig. 2D, arrow). The presence of apoptosis in single cells suggested that in this cocultivation system, stable cell fusions were not required for programmed cell death to occur. The contribution of single cells to the total apoptotic population was minor and was most apparent soon after cocultivation; at 12 h after cocultivation, when relatively few cells were undergoing apoptosis, the number of hypercondensed nuclei in single cells was similar to the number of nuclei in syncytia undergoing apoptosis. By the peak of apoptosis, the majority of apoptotic bodies occurred in syncytia. These histologic studies permitted the earliest detection of apoptotic nuclei and demonstrated that a program of cell death was initiated rapidly after H9 and H9IIIB cells were cocultivated.

To determine whether apoptotic changes affecting the nuclei of H9-H9IIIB cocultures progressed to include digestion of DNA, cocultivations were analyzed for the appearance of oligonucleosomal DNA fragments. Uninfected H9 cells and chronically infected H9IIIB cells were cultured at a ratio of one infected cell to four uninfected cells; cells were harvested throughout a 72-h period, and unintegrated DNA was prepared, electrophoresed on 1% agarose gels, and stained with ethidium bromide. As shown in Fig. 3, the presence of fragmented DNA in discrete oligonucleosome-sized bands typical of apoptosis (Fig. 3A) was detected by agarose gel electrophoresis after 12 h of mixing of cells and increased over 48 to 72 h. These findings indicated that programmed cell death initiated by the coculture included digestion of DNA into oligonucleosome-sized fragments. The onset of apoptosis shortly after the mixing of cells and the digestion of DNA into oligonucleosomal fragments coincided with early events in HIV

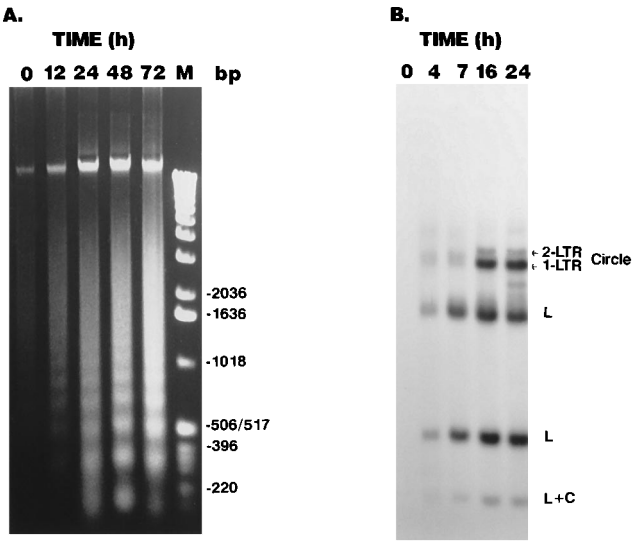


FIG. 3. Degradation of DNA into oligonucleosomal fragments after cocultivation of H9 and H9IIIB cells. (A) H9 and H9IIIB cells were cocultivated at a ratio of one infected cell to four uninfected cells. At the indicated times after cocultivation, 10^6 cells were harvested and unintegrated DNA was prepared and electrophoresed adjacent to a molecular weight marker (M) in 1% agarose gels. The ethidium bromide-stained gel was photographed under UV light. The sizes of the DNA marker bands (in base pairs) are indicated. (B) Unintegrated DNA was prepared from cocultivations at the indicated times, digested with the restriction enzyme *Msc*I, which cuts at positions 2203 and 4135 of the full-length two-long terminal repeat (2-LTR) reverse transcript, and electrophoresed on 0.6% agarose gels. DNA was transferred to Hybond-N+ membranes and probed with *Hin*dIII fragments spanning the HIV genome from position 536 to position 9607. The bands denoted by L represent fragments of 1,932, 2,203, and 5,094 bp generated by *Msc*I from linear HIV DNA, and those denoted by C are derived from the 1- and 2-LTR fragments (indicated by arrows) of 7,751 and 8,386 nucleotides, respectively, and are obtained from circular HIV DNA molecules.

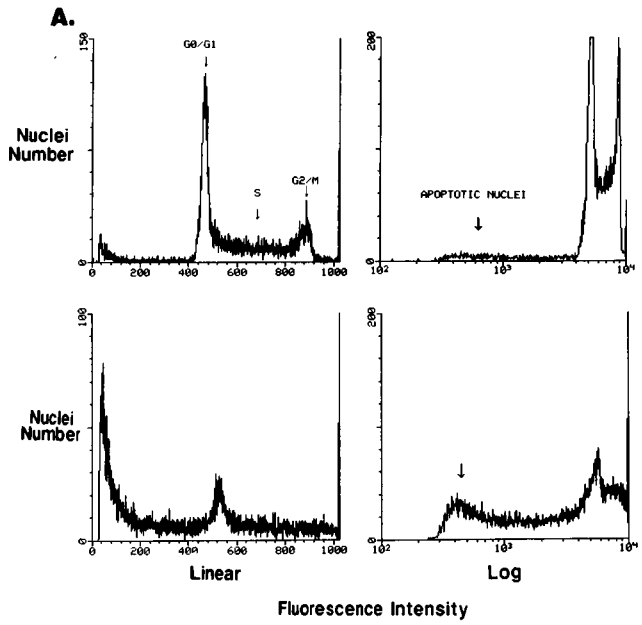
replication. As shown in Fig. 3B, Southern blot analysis of unintegrated DNA revealed that HIV reverse transcription was completed by 4 to 7 h following cocultivation; DNA fragments corresponding to one- and two-long terminal repeat circular forms of HIV DNA, indicative of DNA translocation to the nucleus, were also present, and they increased in amount during the initial 24 h of coculture. This comparison indicated that the early events of HIV replication, including translocation of HIV DNA, took place despite the initiation of apoptosis and nuclear destruction. It is not known whether unintegrated HIV DNA is a substrate for the endonucleases involved in apoptosis-associated DNA degradation; using HIV-specific probes, we have been unable to detect HIV sequences in the oligonucleosomal DNA fragments (data not shown). The delay between the first appearance of condensed nuclei (Fig. 1B) and the onset of oligonucleosomal DNA at 12 h (Fig. 3A) may be attributed to the time required for digestion sufficient to be detected by ethidium bromide staining of DNA in agarose gels.

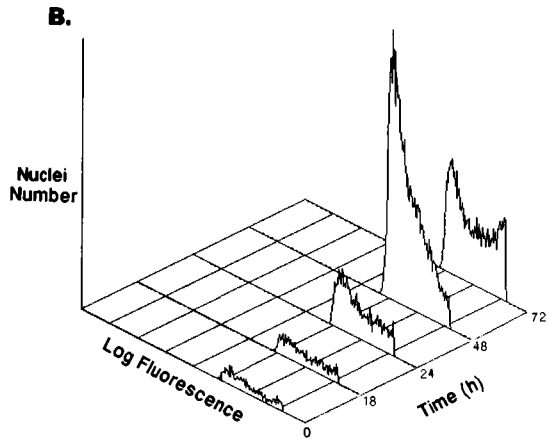
Quantitation of HIV-1-induced programmed cell death. The relative extent of programmed cell death induced by cell-to-cell transmission of HIV was determined by using quantitative fluorescent staining of nuclear DNA to enumerate cells undergoing apoptosis. Three distinct populations of H9 or H9IIIB nuclei were discernible on the basis of DNA content: nuclei with the normal prereplication content of DNA (2N DNA) in G_0/G_1 phase (Fig. 4A); nuclei containing 4N DNA, i.e., nuclei in which DNA had been completely replicated (G_2/M); and nuclei containing amounts of DNA between 2N and 4N (nuclei in S phase). As described by Nicoletti and coworkers (38), the presence of nuclei undergoing apoptosis is identified as a broad peak of nuclei with amounts of DNA less than 2N; to delineate this peak clearly, the fluorescence intensity was adjusted to log scale. Cocultivation of H9 and H9IIIB cells resulted in significant increases in numbers of apoptotic nuclei (Fig. 4A, lower panel), which were absent when the cells were mixed (Fig. 4A, upper panel). Time course studies (Fig. 4B) revealed that apoptotic nuclei were present after 18 to 24 h and increased in number over the 72-h cocultivation period; 24% of the nuclei assayed by this method were apoptotic at 24 h after coculture. The delay in the appearance of condensed nuclei detected by fluorescence-activated cell sorter (FACS) analysis compared with that detected by histologic staining may be attributed to the time necessary for fragmentation of the genome to sub-2N levels for the FACS-based detection.

DNA fragmentation induced by cocultivation could be inhibited by preincubating cells for 20 h with 10 μM $ZnSO_4$, an agent known to prevent DNA fragmentation by a variety of inducers (Fig. 4C). Addition of $ZnSO_4$ at the time of cocultivation did not prevent apoptosis (data not shown), suggesting that the rate of apoptosis induced by cocultivation was more rapid than the rate of inhibition by Zn^{2+}. Preincubation of H9 and H9IIIB cells with other agents reported to inhibit apoptosis, such as *N*-acetyl cysteine (10 mM) and cyclosporin A (5 μg/ml), did not prevent apoptosis due to cell-to-cell virus transmission (Fig. 4C).

DNA content analysis was also performed to investigate the induction of apoptosis in the 12D7 clone of the CEM T-cell leukemia cell line. Because of the aneuploid nature of lymphoblastic cell lines (14), the DNA contents of H9IIIB and 12D7 cells are different. As a result, the DNA histograms of H9IIIB and 12D7 cells were not identical and the positions of the peaks representing the G_0/G_1 and G_2/M phases for H9IIIB were distinct from those for 12D7 (Fig. 5; compare 12D7 with H9IIIB). Nevertheless, at 48 h following coculture, a broad hypodiploid peak similar to that obtained with H9-H9IIIB cocultivations was detectable; decreases in G_0/G_1 and G_2/M peaks for both 12D7 and H9IIIB cell nuclei indicated that apoptosis had occurred for both 12D7 and H9IIIB nuclei (Fig. 5, 12D7 + H9IIIB). The presence of apoptosis was confirmed by identifying oligonucleosome-sized fragments upon analysis of unintegrated DNA from H9IIIB-12D7 cultures (data not shown). Similar DNA fragmentation was observed upon cocultivation of H9IIIB cells with Jurkat cells, and apoptosis was detected by using as donor cells H9 cells chronically infected with the HIV infectious molecular clones pNL4-3 and pNL4-3U35 (*vpu*$^-$) and an HIV molecular clone, pALA-5, with slow growth kinetics (data not shown). These results demonstrated that induction of apoptosis was not restricted to H9 as the recipient cell type or to HIV-1_{IIIB} as the source of the virus.

Env-CD4 interactions, but not new HIV replication, were required for induction of apoptosis. To investigate whether new HIV DNA replication was necessary for apoptosis to occur in this system, parallel cultures were treated with 10 μM zidovudine (AZT). As shown in Fig. 6, analysis of cocultivations revealed no decreases in the relative amounts of apoptotic nuclei in cultures treated with AZT prior to coculture (Fig. 6A; compare +AZT with control). The apoptosis observed in the presence of AZT was not a toxic effect of the drug; as shown in Fig. 6A, treatment of H9 or H9IIIB cells with 10 μM AZT revealed no evidence of drug-induced apoptosis. The presence of oligonucleosome-sized DNA fragments in analyses of unintegrated DNA confirmed the presence of apoptosis in AZT-treated cultures (Fig. 6B). Analyses of unintegrated DNA obtained from H9-H9IIIB cocultures treated with 10 μM AZT revealed no HIV DNA, confirming previously





Time (h)	0	18 h	24 h	48 h	72 h
Apoptotic Fraction	6.43	9.92	24.37	87.7	49.9

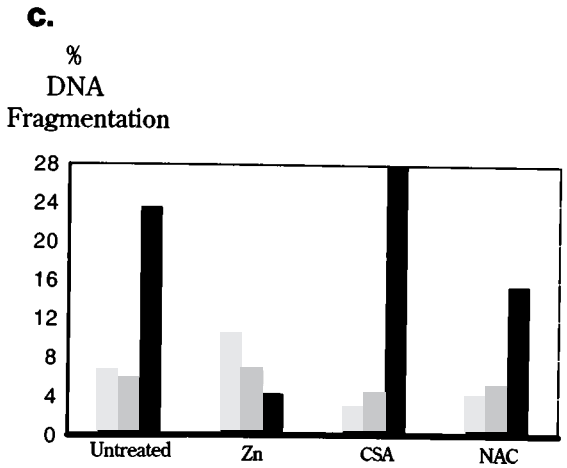


FIG. 4. Detection of HIV-induced apoptosis by FACS analysis. (A) H9 and H9IIIB cells were cocultivated, cells were harvested at time zero (upper panel) and 48 h (lower panel) and lysed, and nuclei were stained with propidium iodide as described in the text. Nuclear DNA content was measured with a FACsort cell sorter using linear and logarithmic fluorescence intensity scales, and the data were analyzed with CELLFIT software. Linear display identified distribution of nuclei in the G_0/G_1, S, and G_2/M phases of the cell cycle, and the log scale identified a broad hypodiploid peak representing accumulation of apoptotic nuclei after 48 h of cocultivation. (B) Time course of accumulation of apoptotic nuclei. The DNA contents of cocultivations were analyzed as described for panel A, and apoptotic nuclei were specifically gated; the relative numbers of apoptotic nuclei present as a function of time after cocultivation are presented. The percentages of total nuclei present in the apoptotic fraction are tabulated below the histograms. (C) Effects of Zn^{2+}, cyclosporin A (CSA), and *N*-acetyl cysteine (NAC) on HIV induction of DNA fragmentation. H9 and H9IIIB cells were preincubated in medium (untreated) or medium containing 10 μM $ZnSO_4$, 5 μg of CSA per ml, or 10 mM NAC for 20 h and then cocultivated (with fresh addition of drug at the time of cocultivation) for 42 h. Cells were harvested, and relative levels of apoptosis were determined as described for panel B. □, H9; ▒, H9IIIB; ■, cocultivation.

published observations (47) that AZT abolished new HIV DNA synthesis in this system (data not shown). These results indicate that synthesis of HIV DNA was not required for apoptosis.

To determine whether Env-CD4 interactions were involved in induction of apoptosis, we investigated the ability of the inhibitor of HIV transmission soluble CD4 (sCD4) to prevent apoptosis in the cell-to-cell transmission model. Preincubation of donor and recipient cells with sCD4, which prevented CD4-Env interactions, cell-cell fusion, and HIV replication (47), also prevented induction of programmed cell death. As shown in Fig. 6A, no increases in numbers of apoptotic nuclei were detected at 48 h after mixing of H9 and H9IIIB cells in the presence of sCD4. These findings suggest that Env-CD4 interactions were necessary for induction of apoptosis. In control experiments, no increases in apoptosis were detected in cultures of H9IIIB cells treated with sCD4 (Fig. 6A), suggesting that CD4 alone cannot trigger this signal in H9IIIB cells.

Marked delay in induction of apoptosis by cell-free HIV infections. The rapid induction of apoptosis caused by cell-to-cell transmission of HIV was inhibited by sCD4, suggesting that Env-CD4 interactions triggered the apoptosis pathway. We investigated whether similar rapid induction of apoptosis occurred when cell-free HIV virions were used as a source of

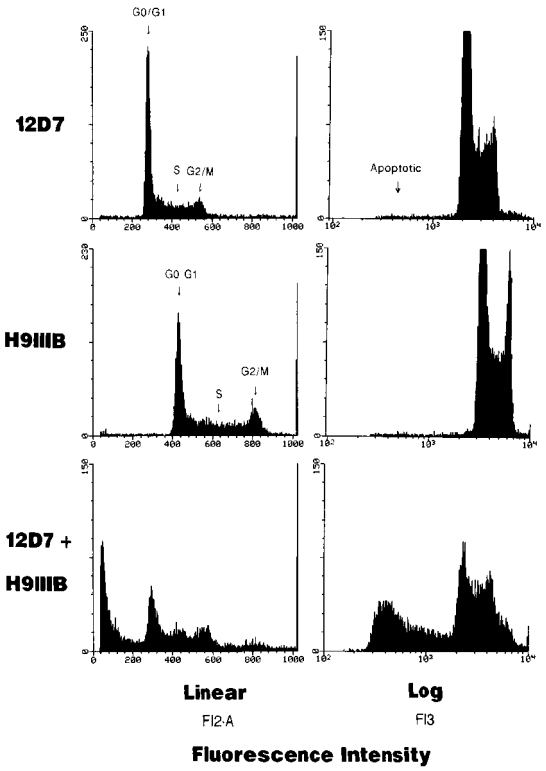


FIG. 5. Induction of apoptosis in 12D7 cells. 12D7 cells and H9IIIB cells were cocultivated at a ratio of 1:4 for 48 h, and apoptosis was detected by FACS DNA content analysis. Data are presented in linear scale to demonstrate differences in positions of G_0/G_1 and G_2/M peaks for the two cell lines. The log scale demonstrates the appearance of apoptotic nuclei (arrow) after cocultivation.

Env protein. The kinetics of induction of apoptosis in cell-to-cell and cell-free HIV infections were compared; 2×10^5 H9 cells were infected with cell-free HIV or chronically infected H9IIIB cells. As shown in Fig. 7A, induction of apoptosis in cell-free HIV infections was markedly delayed compared with that initiated by cell-cell transmission; with concentrated cell-free HIV corresponding to a multiplicity of infection of approximately 0.2, apoptotic nuclei were undetectable until 66 to 88 h after cell-free infections were initiated. As shown in the Western blot in Fig. 7B, the relative amount of inoculum HIV protein used in the cell-free infection was greater than that used in the cocultivation. The amount of cell-free virus (Fig. 7, lane 1:3) represents one-third of the virus inoculum used to infect H9 cells and is approximately equivalent to that present in 3.9×10^5 to 7.8×10^5 H9IIIB cells, which is 8- to 15-fold greater than the number of cells actually used in the parallel cocultivation experiment (5×10^4 H9IIIB cells were used to infect 2×10^5 H9 cells). Thus, despite the presence of 8- to 15-fold more virus protein in the cell-free inoculum, the peak of apoptosis was delayed by 70 h compared with that for the cocultures (Fig. 7A). These data demonstrate that cell-to-cell HIV transmission was much more efficient in inducing apoptosis than were cell-free virions, and they suggest that during in vitro infections with HIV, the apoptosis observed is due primarily to cell-to-cell HIV transmission.

Since apoptosis was not observed in cell-free infections until after one round of HIV replication, a possible explanation for the delay in the kinetics of apoptosis is that programmed cell death is exclusively associated with cell-to-cell transmission and that it was observed in cell-free infections only after the first round of HIV replication, when cell-to-cell HIV transmission takes place. To investigate whether HIV-induced apoptosis could occur in cells infected with virions in the absence of cell-to-cell HIV spread, we initiated infections of H9 cells and blocked cell-to-cell transmission of HIV by the addition of sCD4 to the medium at 2 h after infection. As shown in Fig. 7C, addition of sCD4 after the initial HIV infection did not prevent apoptosis following a cell-free HIV infection. Apoptotic nuclei were detectable at 130 h after infection in the presence or absence of sCD4, although the relative numbers of apoptotic nuclei in sCD4-treated cells were reduced. Light microscopic examination of May-Grunwald-Giemsa-stained cells confirmed the presence of single cells undergoing apoptosis (data not shown). These findings indicate that HIV induced apoptosis even when cell-to-cell transmission was blocked, suggesting that infection by HIV virions was a sufficient, albeit inefficient, inducer of apoptosis.

DISCUSSION

The process of programmed cell death is critical in executing structural changes in development and metamorphosis and in balancing cell populations in multicellular organisms (7). Apoptosis has also been identified as a pathologic response to virus infections by members of the retrovirus, orthomyxovirus, iridovirus, togavirus, picornavirus, parvovirus, chicken anemia virus, herpesvirus, and adenovirus families (5, 11, 17, 22, 27, 35–37, 39, 42, 53, 56). Apoptosis has been reported to occur in HIV-infected cells, but the relationship between HIV replication and the induction of cell death is unclear. We investigated the mode and tempo of cell death produced by synchronous cell-to-cell HIV transmission, for which the kinetics of HIV replication are well defined (8, 47). We found that apoptosis occurred rapidly after coculture of chronically infected and uninfected cells, and a variety of techniques provided complementary data regarding the process of programmed cell death. Microscopic studies detected chromatin condensation in single nuclei, were extremely sensitive in identifying the first nuclei undergoing apoptosis (2 to 8 h), and demonstrated that apoptosis occurred in single cells and in cell fusions; FACS analyses permitted analyses of entire populations and provided approximate quantitations of the extent of apoptosis. Analyses of unintegrated DNA by gel electrophoresis demonstrated that apoptosis proceeds to degradation of DNA into oligonucleo-

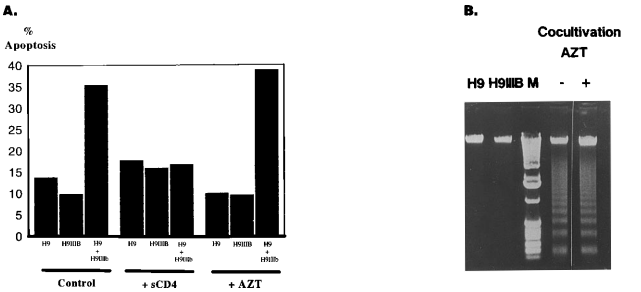


FIG. 6. Effects of sCD4 and AZT on induction of apoptosis. (A) H9 and H9IIIB cells were preincubated in 10 μg of sCD4 per ml, 10 μM AZT, or media for 1 h prior to cocultivation. Cells were harvested at 48 h after cocultivation, nuclear DNA content was quantitated by FACS analysis, and the percentages of total nuclei present in the apoptotic fraction are presented. (B) H9 and H9IIIB cells were treated in the presence or absence of 10 μM AZT as described for panel A; unintegrated DNA prepared from each of the cultures was electrophoresed in 1% agarose gels and stained with ethidium bromide. M is the 1-kb marker described in the legend to Fig. 3.

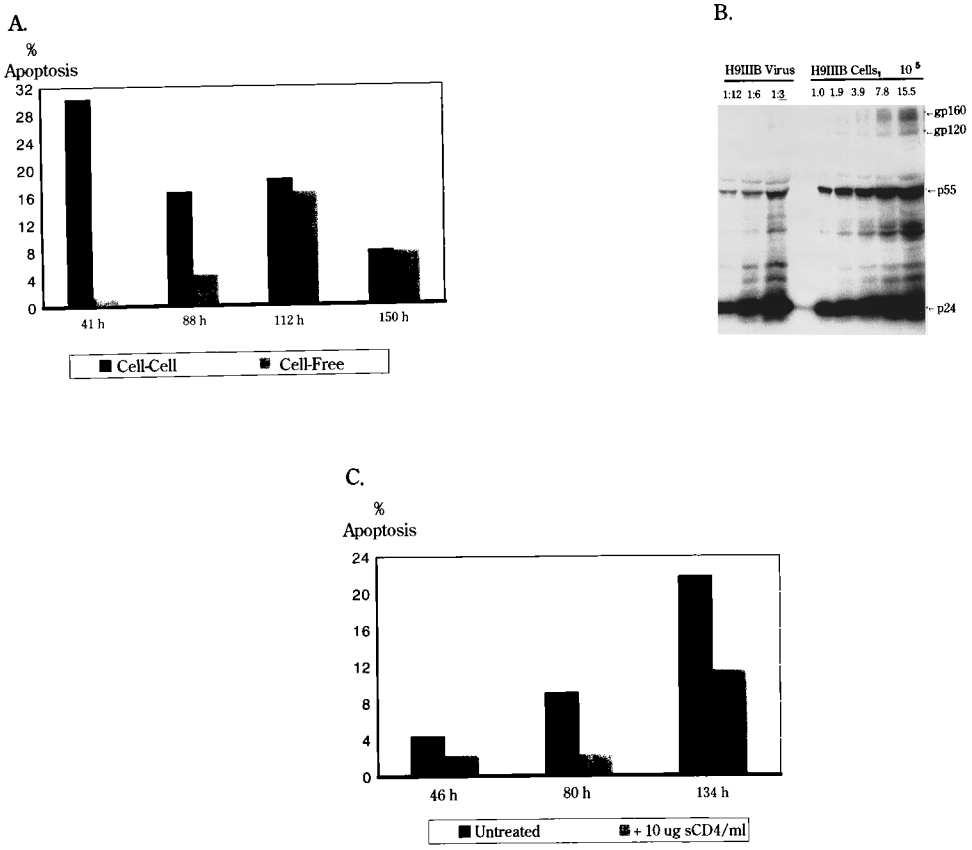


FIG. 7. Induction of apoptosis by cell-to-cell and cell-free HIV transmission. (A) HIV_{IIIB} cell-free virions from chronically infected cells were concentrated and used to infect 2×10^5 H9 cells (multiplicity of infection, approximately 2). In parallel, H9-H9IIIB cocultivation was initiated (2×10^5 H9 cells with 5×10^4 H9IIIB cells), and the percentages of total nuclei undergoing apoptosis were determined by FACS analysis at the indicated times after infection or cocultivation. (B) Determination of relative amounts of HIV protein used in infection and cocultivation. Aliquots of chronically infected cells and of concentrated HIV used for the cocultivation and infection described for panel A were serially diluted, electrophoresed, and immunoblotted as described in the text; the lowest dilution of HIV virions (1:3) corresponds to one-third of the total virus used for the infection described for panel A. The highest dilution of cells (10^5) corresponds to twice the number of H9IIIB cells used for the cocultivation described for panel A. (C) Induction of apoptosis by virions in the absence of cell spread. Cell-free HIV was used to infect H9 cells; at 2 h after infection the virus was removed and the cells were washed and replated in media in the presence or absence of 10 μg of sCD4 per ml. Aliquots of cells were obtained at the indicated times, and percentages of nuclei undergoing apoptosis were determined by FACS analysis.

somal DNA fragments. Although Env-CD4 interactions were required for apoptosis to occur and were blocked by sCD4, no new HIV replication was necessary. These findings support those of Laurent-Crawford and coworkers, who identified Env-mediated induction of apoptosis in cells containing vaccinia virus-expressed Env and CD4 (26), and are consistent with a model in which HIV Env protein cross-links CD4 on the cell surface and triggers apoptosis upon HIV transmission. These findings do not rule out other mechanisms of HIV-induced cell death which rely on virus replication, but they do suggest that these additional mechanisms may take place in cells already committed to cell death.

An unexpected finding of these studies was the marked inefficiency of the induction of apoptosis in cell-free infections compared with the induction in cell-to-cell HIV infections. Differences could not be explained by the amount of HIV protein in virus preparations compared with that in infected cells used to transmit the infection, and they were unlikely to be due to cell-cell fusion alone, since many single cells were observed undergoing apoptosis within 24 to 48 h in cell-cell, but not cell-free, cultures. It is possible that the concentration of Env protein at the apposition of donor and recipient cell membranes is much greater than any ever achieved by points of contact between HIV virions and recipient cell membranes or that it is much more effective in CD4 cross-linking, thereby generating a more potent apoptosis signal. In this regard, the virion inoculum may have downregulated the CD4 receptor and reduced the ability of these cells to become infected, perhaps explaining the reduced amount of apoptosis observed in virion infections in the presence of sCD4 (Fig. 7C). Alternatively, interaction of other cell surface proteins not present on virions may enhance the induction of apoptosis. Nevertheless, the greater efficiency of apoptosis induction by cell-to-cell transmission suggests that during the course of in vitro HIV infections, observed apoptosis is likely to be the product of cell-to-cell HIV transmission.

Studies reported here and elsewhere demonstrate a central role for Env-CD4 interactions in initiating apoptosis, but the pathway of programmed cell death subsequent to the Env-CD4 interaction has not been elucidated. Multiple independent pathways culminating in apoptosis have been identified (7, 45, 51), and it is unclear whether HIV-induced cell death occurs by a known triggering mechanism or by a pathway yet to be defined. Whether HIV proteins other than Env participate in, or modulate, the apoptotic process is unknown; recent reports suggest that expression of the HIV and human T-cell leukemia virus type 1 transactivator proteins Tat and Tax, respectively, may protect (59), accelerate (55), or induce apoptosis (28, 58).

In our cocultivation experiments, some cell fusions showed marked degenerative changes in nuclear appearance within 2 to 8 h of coculture, while others contained nuclei that appeared normal even after 32 h of cocultivation (Fig. 1). In addition, HIV infections of H9 cells characteristically yield survivor cells chronically expressing infectious virus without apoptosis, demonstrating that some infected cells can escape cell death. We are presently investigating the expression of viral and cellular genes involved in apoptosis in the rapidly apoptotic and the nondegenerating nuclei to characterize the pathway of apoptosis occurring after HIV infection.

One consequence of rapid induction of apoptosis is that the expression of HIV RNA and protein occurs in cells undergoing marked changes in nuclear organization. It is not clear how nuclear destruction affects the functions of HIV proteins such as Tat, Rev, and Vpr. Tat and Rev have been associated with intact nucleoli, which are destroyed early in the apoptotic process. It will be of interest to determine whether the transactivating functions of Tat and the splicing and RNA transport activities attributed to Rev (20) still occur in nuclei undergoing programmed cell death.

$CD4^+$ T-cell depletion remains a key unexplained characteristic of acquired immunodeficiency, which predisposes the patient to lethal infectious and neoplastic complications of AIDS and for which no therapy is effective. Programmed cell death has been postulated as a potential mechanism for $CD4^+$ T-cell loss in HIV-infected persons (15, 33, 43, 52) because lymphocytes from infected persons undergo accelerated apoptosis after cultivation in vitro. It is of interest that no accelerated apoptosis was detected in lymphocytes cultured from HIV-infected chimpanzees, animals which become viremic but show no CD4 T-cell decline and no disease progression after HIV infection (16, 48). No correlation between susceptibility to apoptosis in vitro and CD4 decline or AIDS disease progression has been observed (34), and apoptosis has been observed in peripheral blood mononuclear cells cultured from patients with chronic illnesses other than HIV infection, including leukemia and systemic lupus erythematosus (9, 21, 46), suggesting that accelerated programmed cell death ex vivo may represent a nonspecific defect in peripheral blood mononuclear cell longevity or a result of imperfect cultivation conditions and not a specific pathologic consequence of HIV infection. Recently, apoptotic cells have been identified in lymph nodes of HIV-infected children and simian immunodeficiency virus-infected primates, primarily among bystander cells and not virus-infected cells (13). Our finding that infected cells efficiently and rapidly induce apoptosis following cell-to-cell transmission of HIV suggests apoptosis may contribute to progressive lymphocyte depletion within organs such as lymph nodes, in which relatively few infected cells are present (44) but extensive cell-cell interactions are possible. In addition, direct T-cell killing has been inferred in explaining CD4 cell decline in HIV infection and the recovery of CD4 cells after treatment with HIV protease inhibitors (18, 54). In this regard, we have detected rapid induction of apoptosis after cocultivation of peripheral blood mononuclear cells from uninfected donors with chronically infected H9 cells (unpublished data), demonstrating that HIV-associated apoptosis is efficiently induced in lymphocytes as well as in T-cell lines. By continuing to characterize the multiple mechanisms that result in the elimination of $CD4^+$ cells, including apoptosis, we may identify novel approaches to prevent lymphocyte depletion and prolong the disease-free period following the initial exposure to HIV.

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REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59:**284–291.
2. **Ameisen, J. C.** 1992. Programmed cell death and AIDS: from hypothesis to experiment. Immunol. Today **13:**388–391.
3. **Banda, N., J. Bernier, D. K. Kurahara, R. Kurrle, N. Haigwood, R.-P. Sekaly, and T. H. Finkel.** 1992. Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. J. Exp. Med. **176:**1099–1106.
4. **Bergeron, L., and J. Sodroski.** 1992. Dissociation of unintegrated viral DNA accumulation from single-cell lysis induced by human immunodeficiency virus type 1. J. Virol. **66:**5777–5787.
5. **Clem, R. J., and L. K. Miller.** 1994. Control of programmed cell death by the baculovirus genes *p35* and *iap*. Mol. Cell. Biol. **14:**5212–5222.
6. **Cloyd, M. W., and W. S. Lynn.** 1991. Perturbation of host-cell membrane is a primary mechanism of HIV cytopathology. Virology **181:**500–501.
7. **Collins, M. K. L., and A. L. Rivas.** 1993. The control of apoptosis in mammalian cells. Trends Biochem. Sci. **18:**307–309.
8. **Dimitrov, D. S., R. L. Willey, H. Sato, L.-J. Chang, R. Blumenthal, and M. A. Martin.** 1993. Quantitation of human immunodeficiency virus type 1 infection kinetics. J. Virol. **67:**2182–2190.
9. **Emlen, W., J. Niebur, and R. Kadera.** 1994. Accelerated in vitro apoptosis of lymphocytes from patients with systemic lupus erythematosus. J. Immunol. **152:**3685–3692.
10. **Englund, G., M. D. Hoggan, T. S. Theodore, and M. A. Martin.** 1991. A novel HIV-1 isolate containing alterations affecting the NF-κB element. Virology **181:**150–157.
11. **Fesq, H., M. Bacher, M. Nain, and D. Gemsa.** 1994. Programmed cell death (apoptosis) in human monocytes infected by influenza A virus. Immunobiology **190:**175–182.
12. **Finkel, T. H., and N. K. Banda.** 1994. Indirect mechanisms of HIV pathogenesis: how does HIV kill T cells? Curr. Opin. Immunol. **6:**605–615.
13. **Finkel, T. H., G. Tudor-Williams, N. K. Banda, M. F. Cotton, T. Curiel, C. Monks, T. W. Baba, R. M. Ruprecht, and A. Kupfer.** 1995. Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. Nat. Med. **1:**129–138.
14. **Glukhova, L. A., I. A. Kiseleva, M. N. Korneeva, D. N. Nosik, A. A. Kushch, S. E. Mamaeva, and B. Ashe.** 1991. Karyological approach to the identification of true cell lines susceptible to the human immunodeficiency virus (HIV). Biomed. Sci. **2:**293–297.
15. **Groux, H., G. Torpier, D. Monte, Y. Mouton, A. Capron, and J. C. Ameisen.** 1992. Activation-induced cell death by apoptosis. J. Exp. Med. **175:**331–340.
16. **Heeney, J., R. Jonker, W. Koornstra, R. Dubbes, H. Niphuis, A. M. Di Rienzo, M. L. Gougeon, and L. Montagnier.** 1993. The resistance of HIV-infected chimpanzees to progression to AIDS correlates with absence of HIV-related T-cell dysfunction. J. Med. Primatol. **22:**194–200.
17. **Hinshaw, V. S., C. W. Olsen, N. Dybdahl-Sissoko, and D. Evans.** 1994. Apoptosis: a mechanism of cell killing by influenza A and B viruses. J. Virol. **68:**3667–3673.
18. **Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz.** 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature (London) **373:**123–126.
19. **Hoggan, M. D., R. R. O'Neill, and C. A. Kozak.** 1986. Nonecotropic murine leukemia viruses in BALB/c and NFS/N mice: characterization of the BALB/c *Bxv-1* provirus and the single NFS endogenous xenotrope. J. Virol. **60:**980–986.
20. **Jeang, K.-T., and A. Gatignol.** 1994. Comparison of regulatory features in primate lentiviruses. Curr. Top. Microbiol. Immunol. **188:**123–144.
21. **Kalvelyte, A., and A. Pabrezaite.** 1994. Phenomena of spontaneous proliferation, differentiation, and apoptosis in primary cultures of leukemic lymphocytes. Exp. Cell Res. **111:**344–352.
22. **Kawanishi, M.** 1993. Epstein-Barr virus induces fragmentation of chromosomal DNA during lytic infection. J. Virol. **67:**7654–7658.
23. **Klimkait, T., K. Strebel, M. D. Hoggan, M. A. Martin, and J. M. Orenstein.** 1990. The human immunodeficiency virus type 1-specific protein *vpu* is required for efficient virus maturation and release. J. Virol. **64:**621–629.
24. **Koga, Y., K. Nakamura, M. Sasaki, G. Kimura, and K. Nomoto.** 1994. The difference in gp160 and gp120 of HIV type 1 in the induction of CD4 downregulation preceding single-cell killing. Virology **201:**137–141.
25. **Laurent-Crawford, A. G., B. Krust, S. Muller, Y. Riviere, M.-A. Rey-Cuille, J.-M. Bechet, L. Montagnier, and A. G. Hovanessian.** 1991. The cytopathic effect of HIV is associated with apoptosis. Virology **185:**829–839.
26. **Laurent-Crawford, A. G., B. Krust, Y. Riviere, C. Desgranges, S. Muller, M. P. Kieny, C. Dauguet, and A. G. Hovanessian.** 1993. Membrane expres-

sion of HIV envelope glycoproteins triggers apoptosis in CD4 cells. AIDS Res. Hum. Retroviruses **9:**761–773.
27. **Levine, B., Q. Huang, J. T. Isaacs, J. C. Reed, D. E. Griffin, and J. M. Hardwick.** 1993. Conversion of lytic to persistent alphavirus infection by the bcl-2 cellular oncogene. Nature (London) **361:**739–742.
28. **Li, C. J., D. J. Friedman, C. Wang, V. Metelev, and A. B. Pardee.** 1995. Induction of apoptosis in uninfected lymphocytes by HIV-1 Tat protein. Science **268:**429–431.
29. **Li, P., and C. J. Burrell.** 1992. Synthesis of human immunodeficiency virus DNA in a cell to cell transmission model. AIDS Res. Hum. Retroviruses **8:**253–259.
30. **Lu, Y.-Y., Y. Koga, K. Tanaka, M. Sasaki, G. Kimura, and K. Nomoto.** 1994. Apoptosis induced in $CD4^+$ cells expressing gp160 of human immunodeficiency virus type 1. J. Virol. **68:**390–399.
31. **Maldarelli, F., N. W. King, and M. J. Yagi.** 1987. Effects of cytoskeletal disrupting agents on replication of mouse mammary tumor virus. Virus Res. **7:**281–295.
32. **Martin, S. J., P. M. Matear, and A. Vyakarnam.** 1994. HIV infection of human CD4+ T cells in vitro. Differential induction of apoptosis in these cells. J. Immunol. **152:**330–342.
33. **Meyaard, L., S. A. Otto, R. R. Jonker, M. J. Mijnster, R. P. M. Keet, and F. Miedema.** 1992. Programmed cell death of T cells in HIV-1 infection. Science **257:**217–219.
34. **Meyaard, L., S. A. Otto, I. P. M. Keet, M. T. L. Roos, and F. Miedema.** 1992. Programmed cell death of T cells in human immunodeficiency virus infection. No correlation with progression to diease. J. Clin. Invest. **93:**982–988.
35. **McCarthy, S. A., H. S. Symonds, and T. Van Dyke.** 1994. Regulation of apoptosis in transgenic mice by simian virus 40 T antigen-mediated inactivation of p53. Proc. Natl. Acad. Sci. USA **91:**3979–3983.
36. **Morey, A. L., D. J. Ferguson, and K. A. Fleming.** 1993. Ultrastructural features of fetal erythroid precursors infected with parvovirus B19 in vitro: evidence of cell death by apoptosis. J. Pathol. **169:**213–220.
37. **Mountz, J. D., and N. Talal.** 1993. Retroviruses, apoptosis and autogenes. Immunol. Today **14:**532–536.
38. **Nicoletti, I., G. Migliorati, M. C. Pagliacci, F. Grignani, and C. Riccardi.** 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Methods **139:** 271–279.
39. **Noteborn, M. H. M., D. Todd, C. A. J. Verschueren, H. W. F. M. de Gauw, W. L. Curran, S. Veldkamp, A. J. Douglas, M. S. McNulty, A. J. van der Eb, and G. Koch.** 1994. A single chicken anemia virus protein induces apoptosis. J. Virol. **68:**346–351.
40. **Oberhammer, F. A., G. Fritsch, M. Schmid, M. Pavelka, D. Printz, T. Purchi, H. Lassmann, and R. Schulte-Hermann.** 1993. Condensation of the chromatin at the membrane of an apoptotic nucleus is not associated with activation of an endonuclease. J. Cell Sci. **104:**314–326.
41. **Oberhammer, F. A., J. W. Wilson, C. Dive, I. D. Morris, J. A. Hickman, A. Wakeling, P. R. Walker, and M. Sikorska.** 1993. Apoptotic death in epithelial cells: cleavage of DNA to 300 and 50 kilobase fragments prior to internucleosomal fragmentation. EMBO J. **12:**3679–3684.
42. **Ohno, K., Y. Okamoto, T. Miyazawa, T. Mikami, T. Watari, G. R. Goitsuka, R. Tsujimoto, and A. Hasegawa.** 1994. Induction of apoptosis in a T lymphoblastoid cell line infected with feline immunodeficiency virus. Arch. Virol. **135:**153–158.
43. **Oyaizu, N., T. W. McCloskey, M. Coronesi, N. Chirmule, V. S. Kalyanaraman, and S. Pahwa.** 1993. Accelerated apoptosis in peripheral blood mononuclear cells (PBMCs) from human immunodeficiency virus type-1 infected patients and in CD4 cross-linked PBMCs from normal individuals. Blood **82:**3392–3400.
44. **Panteleo, G., C. Graziosi, and A. S. Fauci.** 1993. The role of lymphoid organs in the pathogenesis of HIV infection. Semin. Immunol. **5:**157–163.
45. **Reed, J. C.** 1994. Bcl-2 and the regulation of programmed cell death. J. Cell Biol. **124:**1–6.
46. **Robertson, L. E., and W. Plunkett.** 1993. Apoptotic cell death in chronic lymphocytic leukemia. Leuk. Lymphoma **11**(Suppl. 2)**:**71–74.
47. **Sato, H., J. Orenstein, D. Dimitrov, and M. A. Martin.** 1992. Cell-to-cell spread of HIV-1 occurs within minutes and may not involve the participation of virus particles. Virology **186:**712–724.
48. **Schuitemaker, H., L. Meyaard, N. A. Kootstra, R. Dubbes, S. A. Otto, M. Tersmette, J. L. Heeney, and F. Miedema.** 1993. Lack of T cell dysfunction and programmed cell death in human immunodeficiency virus type 1-infected chimpanzees correlates with absence of monocytotropic variants. J. Infect. Dis. **168:**1140–1147.
49. **Somasundaran, M., and H. L. Robinson.** 1987. A major mechanism of human immunodeficiency virus-induced cell killing does not involve cell fusion. J. Virol. **61:**3114–3119.
50. **Strebel, K., T. Klimkait, and M. A. Martin.** 1988. A novel gene of HIV-1, vpu, and its 16-kilodalton product. Science **241:**1221–1223.
51. **Sun, D. Y., S. Jiang, L.-M. Zheng, D. M. Ojcius, and J. D.-E. Young.** 1994. Separate metabolic pathways leading to DNA fragmentation and apoptotic chromatin condensation. J. Exp. Med. **179:**559–568.
52. **Terai, C., R. S. Kornbluth, C. D. Pauza, D. D. Richman, and D. A. Carson.** 1991. Apoptosis as a mechanism of cell death in cultured T lymphoblasts acutely infected with HIV-1. J. Clin. Invest. **87:**1710–1715.
53. **Ubol, S., P. C. Tucker, D. E. Griffin, and J. M. Hardwick.** 1994. Neurovirulent strains of Alphavirus induce apoptosis in bcl-2-expressing cells: role of a single amino acid change in the E2 glycoprotein. Proc. Natl. Acad. Sci. USA **91:**5202–5206.
54. **Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, and B. H. Hahn.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature (London) **373:**117–122.
55. **Westendorp, M. O., R. Frank, C. Ochsenbauer, K. Stricker, J. Dhein, H. Walczak, K. M. Debatin, and P. H. Krammer.** 1995. Sensitization of T-cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. Nature (London) **375:**497–500.
56. **White, E.** 1993. Regulation of apoptosis by the transforming genes of the DNA tumor virus adenovirus. Proc. Soc. Exp. Biol. Med. **204:**30–39.
57. **Wyllie, A. H.** 1980. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature (London) **284:**555–556.
58. **Yamada, T., S. Yamaoka, T. Goto, M. Nakai, Y. Tsujimoto, and M. Hatanaka.** 1994. The human T-cell leukemia virus type I Tax protein induces apoptosis which is blocked by the Bcl-2 protein. J. Virol. **68:**3374–3379.
59. **Zauli, G., D. Gibellini, D. Miliani, M. Mazzoni, P. Borgatti, M. LaPlaca, and S. Capitani.** 1993. Human immunodeficiency virus type 1 tat protein protects lymphoid, epithelial, and neuronal cell lines from death by apoptosis. Cancer Res. **53:**4481–4485.