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The Human Immunodeficiency Virus Type 1 *vpr* Gene Arrests Infected T Cells in the G_2 + M Phase of the Cell Cycle

JEREMY B. M. JOWETT, VICENTE PLANELLES, BETTY POON, NEIL P. SHAH, MENG-LIANG CHEN, AND IRVIN S. Y. CHEN*

Department of Microbiology & Immunology,[1] Division of Hematology-Oncology, Department of Medicine,[2] and Jonsson Comprehensive Cancer Center,[3] UCLA School of Medicine, Los Angeles, California 90095-1678

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Human immunodeficiency virus type 1 (HIV-1) infection causes profound immunological defects in afflicted patients. Various mechanisms have been proposed to account for the immune dysfunction in AIDS ultimately leading to loss of $CD4^+$ T cells, including HIV-1 envelope-mediated syncytium formation, apoptosis, and cytokine modulation. Here we present results which suggest a novel hypothesis for T-cell dysfunction. We show, using HIV-1 bearing a novel cell surface reporter gene, that infected cells are unable to progress normally through the cell cycle and became arrested in the G_2 + M phase. Furthermore, we identify the HIV-1 *vpr* gene product as being both necessary and sufficient for eliciting this cell cycle arrest. Cell cycle arrest induced by Vpr correlates with an increase in the hyperphosphorylated (inactive) form of the cyclin-dependent serine/threonine kinase CDC2, consistent with an arrest of cells at the boundary of G_2 and M.

Human immunodeficiency virus type 1 (HIV-1), the etiological agent of AIDS, can infect and ultimately incapacitate the immune system. The immunopathogenesis associated with HIV-1 infection has been closely related to the functional abnormalities and quantitative depletion of CD4 T lymphocytes, the primary target of the virus (11). Both virological and immunological mechanisms have been proposed to account for this immune dysfunction (10, 43, 52, 53). Early studies focused on cytopathogenesis by direct killing of target cells following infection. Among the virally mediated mechanisms investigated were toxicity as a result of accumulation of unintegrated viral DNA following reverse transcription (51), lethal membrane permeability changes resulting from virus particles budding at the surface of the infected cell (11), and terminal differentiation causing a shortened life span of the CD4 T lymphocyte (66). Another possible cause of cell death was noted in in vitro studies in which HIV-1-infected cells fused with neighboring cells by a viral envelope/receptor-mediated interaction to form multinucleated cells (syncytia) that die within 48 h (29, 30, 56). The emergence of the syncytium-inducing strains coincided with the decline in CD4 cell number and loss of immune function, but the cause and effect have yet to be established (5, 62). Furthermore, evidence of syncytium formation has not been found in vivo; therefore, its role in immunopathogenesis remains unclear (53). A type of programmed cell death known as apoptosis has also been proposed as a cause of cell death, which occurs through inappropriate receptor signaling (2, 4). Fresh peripheral blood lymphocytes (PBL) from HIV-1-infected patients had a greater propensity to undergo apoptosis following stimulation in vitro than those from healthy individuals (15, 32). HIV-1 infection in vitro has also been observed to induce apoptosis in certain T-cell lines (14, 25, 61), although this type of death was linked to envelope/receptor-mediated cell fusion.

In an effort to learn more about the consequences of HIV-1 infection in individual cells, we designed a system to examine the effect of HIV-1 early after infection. To distinguish infected from uninfected cells, we used a novel cell surface marker to tag HIV-1-infected cells (47). We demonstrate that individual cells infected with HIV-1 are impaired in their ability to progress through the cell cycle. More specifically, HIV-1 infection results in arrest of the target cell in the second gap (G_2) phase or early in mitosis (M). Furthermore, we demonstrate that the virally encoded *vpr* gene product is both necessary and sufficient for the observed cell cycle perturbation. From these results, we propose an alternative model for HIV-1-induced immunodeficiency.

MATERIALS AND METHODS

Cells. SupT1 cells (human $CD4^+$ T cells), derived from a non-Hodgkin's lymphoma (AIDS Research and Reference Reagent Program, National Institutes of Health), were passaged in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; Gemini, Calabasas, Calif.), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 mM glutamine (Irvine Scientific, Santa Ana, Calif.). MT-2 cells, a human T-cell lymphotrophic virus type 1 (HTLV-1)-transformed T-cell line (AIDS Research and Reference Reagent Program), were propagated in Iscove's medium (Gibco-BRL) supplemented with 10% FCS and antibiotics, as described above. HeLa (human epithelial), COS, CV-1 (African green monkey kidney), and NIH 3T3 (murine embryonic) cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% calf serum (Gemini).

PBL were obtained from normal donors by venipuncture, isolated by centrifugation over Ficoll-Hypaque (Pharmacia, Sweden), and depleted of macrophages by adherence to plastic for 4 h. The lymphocytes were then cultured in the presence of phytohemagglutinin (PHA; 5 μg/ml; Sigma Chemical Co., St. Louis, Mo.) for 3 days prior to infection. The culture was enriched for the $CD4^+$ population by negative selection panning (64) with anti-CD8 (OKT8) and anti-CD11b (OKM1) antibodies. These antibodies were prepared from hybridoma cell lines obtained from the American Type Culture Collection (OKT8 and OKM1). Levels of CD4 cells were determined pre- and postpanning by staining with anti-CD4 antibodies (Becton Dickinson, San Jose, Calif.) conjugated to fluorescein isothiocyanate (FITC), and flow cytometry was carried out as described below. Following infection, these cells were cultured in RPMI supplemented with 10% FCS and 30 U of recombinant interleukin 2 (Amgen, Thousand Oaks, Calif.) per ml with antibiotics.

Virus and infections. Viral stocks of HIV-1_{NL4-3} and NL-Thy were generated by electroporation of MT-2 cells. Briefly, 5×10^6 cells were collected at mid-log phase, pelleted at $300 \times g$, and resuspended in electroporation medium (RPMI with 20% FCS). Plasmid pNL4-3 (1) or pNL-Thy (10 μg) (see below) was added, incubated on ice for 5 min, electroporated at 960 μF and 300 V, incubated on ice for a further 5 min, and finally resuspended in 20 ml of growth medium. At 6 to 8 days posttransfection, culture supernatant was harvested and assayed for in-

* Corresponding author. Mailing address: 11-934 Factor, Division of Hematology-Oncology, Department of Medicine, UCLA School of Medicine, Los Angeles, CA 90095-1678. Phone: (310) 825-4793. Fax: (310) 825-6192.

fectious virus titer by limiting dilution assay and for HIV-1 core antigen (p24) by enzyme-linked immunosorbent assay (ELISA; Coulter, Hialeah, Fla.). Cultures of MT-2 cells were also mock transfected with sterile TE buffer, and supernatant was harvested at the same time for use in mock infections. Viral stocks were stored at −70°C until use. Target cells (5×10^6) were infected by suspension in viral stock and 10 μg of Polybrene (Sigma) per ml at 37°C for 1 h, with gentle agitation. Cells were washed and resuspended at 5×10^5/ml in growth medium. Mock-infected cultures were generated as above except that the inoculum was derived from mock-transfected MT-2 cells, as described above.

The Vpr-X mutant virus was based on NL-Thy except that plasmid pNL-Thy was digested with *Eco*RI (nucleotide position 132 of the *vpr* open reading frame), blunt-ended by filling in with the DNA polymerase I Klenow fragment, and religated according to standard procedures (49). The resulting insertion/frameshift replaced the carboxy-terminal 33 residues of the 96-amino-acid Vpr protein with the sequence NSATTAVYPFQNWVST (shown in single-letter amino acid code). Viral stocks were prepared as for NL-Thy.

Inhibitors of viral replication were added to culture media where stated in the text as follows: soluble CD4 (sCD4) was used at 10 μg/ml, azidothymidine (AZT) was used at 5 mM, and the protease inhibitor A77003 (kind gift of Andrew Kaplan, UCLA) was used at 3 μM. sCD4 and A77003 were added at 3 h postinfection, while AZT was added at 12 h to allow completion of reverse transcription.

Plasmids. The construction of pNL-Thy has been described in detail elsewhere (HIV-Thy-1) (47). Briefly, the *nef* open reading frame of pNL4-3 (1) was deleted from the *Xho*I to *Kpn*I sites (nucleotide positions 8888 and 9006, respectively) and replaced with the coding sequence for the murine thymocyte surface antigen Thy 1.2 (13). This surface antigen was selected for its small size (600 bp), its efficient surface expression, and the availability of specific antibodies suitable for detection by flow cytometry (no cross-reactivity with the human counterpart).

The expression plasmids were constructed to contain the *thy1.2* (13) and the *vpr* (derived from HIV-1_{NL4-3}) open reading frames, both driven by tandem cytomegalovirus (CMV) immediate-early promoter transcription units. Briefly, an *Spe*I-*Xho*I fragment of pCDM8 (Invitrogen, San Diego, Calif.) containing the CMV immediate-early promoter was transferred to the *Spe*I and *Xho*I sites of pNL-Thy (47) to create plasmid NLCMVThy. A *Pst*I digest of NLCMVThy yielded a fragment containing the CMV promoter, the *thy1.2* open reading frame, and the HIV-1_{NL4-3} 3′ long terminal repeat (LTR) sequences, which was transferred into Bluescript II KS Plus (Stratagene, La Jolla, Calif.) cut with *Pst*I to create the intermediate plasmid BSCMVThy. A *Sca*I-*Sac*I fragment that contained the *vpr* open reading frame was recovered from the digest of pNL4-3 (1). This was first cloned into the *Sma*I and *Sac*I sites of plasmid pGEM 7Zf(−) (Promega, Madison, Wis.) to create plasmid pGEMVpr. The *vpr* open reading frame (pGEMVpr cut with *Xho*I and *Nsi*I) was then subcloned into pCDM8 digested with *Xho*I and *Pst*I to give plasmid CDM8Vpr.

To create the dual expression plasmid BSVprThy and the control plasmid BSThy, an *Nru*I-*Bam*HI fragment of either CDM8Vpr or pCDM8 containing the CMV promoter and simian virus 40 transcription termination sequences with or without the *vpr* open reading frame was cloned into the BSCMVThy intermediate plasmid cut with *Not*I (blunt-ended by filling in with Klenow) and *Bam*HI. The plasmid containing the mutant *vpr* (BSVprXThy) was constructed by digestion of the plasmid with *Eco*RI and filling in. The vectors used in some of the experiments shown in Fig. 4 and 5 contained an untranslated intron of the CMV immediate-early promoter (derived from pCMV-Thy-1 [47]) that was found to increase expression efficiency in some cell types (data not shown). The dual expression vector carrying the human *cdc2* gene (BSCDC2Thy) was constructed from pOB231 (kindly provided by Paul Nurse) (27), digested with *Eco*RI, and blunt-ended with Klenow and *Bam*HI. This *cdc2*-containing fragment was cloned into the *Eco*RV and *Bam*HI sites of Bluescript II KS Plus to create BSCDC2. A *Cla*I-*Not*I fragment of BSCDC2 was then cloned into the dual expression vector (carrying CMV introns, as described above) in place of *vpr*. The single *vpr* expression vector BSVpr was created from BSVprThy; the Thy 1.2 expression cassette was deleted by *Mun*I digestion and religation. All cloning steps described followed standard procedures described elsewhere (49).

Plasmid DNA was prepared for transfection by purification on an anion-exchange resin (Qiagen, Chatsworth, Calif.), in accordance with the manufacturer's protocol. Plasmid DNA (10 μg) was added to 5×10^6 cells in electroporation medium. The electroporation conditions for SupT1 cells were as described for MT-2 cells. For COS and HeLa cells, the electroporation parameters were 250 V at 960 μF. Culture medium was replaced at 24 h posttransfection. Cells were harvested at 48 h posttransfection and stained for expression of Thy 1.2 and DNA content.

Flow cytometry. Detection of cells bearing the surface marker Thy 1.2 was done as follows. Cells (10^6) were harvested and stained in 100 μl of Thy 1.2 FITC-conjugated monoclonal antibody (Caltag) diluted 1:200 in fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline [PBS] with 2% FCS and 0.01% sodium azide) and incubated for 20 min on ice. An additional sample of cells was stained with the isotype antibody immunoglobulin G2b (IgG2b)-FITC (Caltag) to control for nonspecific background antibody binding. The cells were then washed and resuspended in FACS buffer containing propidium iodide (PI) (1 μg/ml) and acquired on a FACScan flow cytometer (Becton Dickinson) equipped with a 15-mW air-cooled 488-nm argon ion laser. Green FITC fluorescence was collected after a 530/30-nm band pass filter and displayed on a four-decade log scale. Orange PI fluorescence was collected after a 585/42-nm band pass filter and displayed similarly. Electronic compensation was used among the fluorescence channels to remove residual spectral overlap. A minimum of 5,000 events were collected for each sample. Data analysis was performed with Lysis II software (Becton Dickinson). Since dead cells cannot exclude PI, they were removed from the calculation of percent Thy 1.2^+ cells by gating on the low angle (forward scatter) versus orange fluorescence intensity.

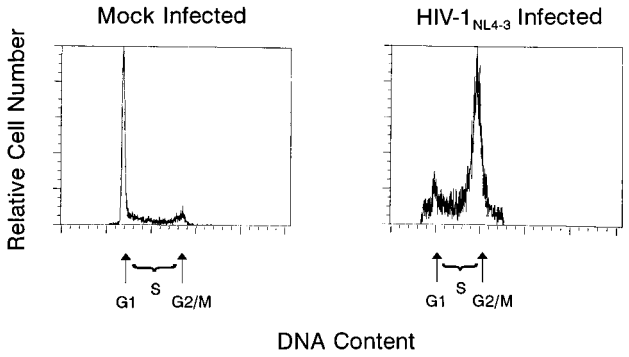


FIG. 1. Infection of SupT1 cells with HIV-1_{NL4-3} causes cell cycle perturbation. DNA content of nuclei from mock-infected and HIV-1_{NL4-3}-infected SupT1 cells (6.9 μg of p24; day 4 postinfection) stained with propidium iodide and analyzed by flow cytometry. The histogram indicates relative proportions of cells in G_1, S, and G_2 + M phases of the cell cycle by relative fluorescence intensity of the stained nuclei (*x* axis).

The method of double staining for surface antigen and DNA content of cells was adapted from the technique of Schmid et al. (50). Briefly, 10^6 cells were harvested and stained as described above for Thy 1.2 surface antigen. After washing, the cells were fixed in PBS with 0.3% paraformaldehyde for 1 h on ice and permeabilized in 0.2% Tween 20 (Bio-Rad) in PBS on ice for 15 min, and the DNA was finally stained in FACS buffer containing propidium iodide (10 μg/ml) and RNase A (11.25 Kunitz units) for 30 min at 4°C. At least 5,000 events were collected, as described above, except that orange PI fluorescence was displayed on a linear scale.

Data analysis and acquisition were performed with both Lysis II and Cellfit software (Becton Dickinson). Samples were gated to exclude debris and clumps, and electronic compensation was used to remove residual spectral overlap. Gates for distinction between Thy 1.2^- and Thy 1.2^+ cells were set based on control stained populations to channel 15 to 20 of FITC relative fluorescence intensity. The mathematical model SOBR (sum of broadened rectangles) was used to calculate the proportions of cells in the G_1, S, and G_2 + M phases of the cell cycle by using Cellfit. These values were in agreement with those obtained with Lysis II software when the S-phase events were divided equally between the G_1 and G_2 + M phases, based on peak channels of fluorescence intensity. For simplicity, the G_1 and G_2 + M values and calculated G_1/G_2 + M ratios from Lysis II have been provided.

Cell lysis and protein analysis. HeLa cells were lysed at 4°C in lysis buffer (Hanks' balanced salt solution with 1% Nonidet P-40, 50 mM β-glycerophosphate, 10 mM NaF, 1% aprotinin, 1 mM sodium orthovanadate, and 1 μg of leupeptin per ml), and protein concentrations were estimated by the Bio-Rad protein assay according to the manufacturer's protocol. Equivalent amounts of protein per sample were subjected to electrophoresis on sodium dodecyl sulfate–15% polyacrylamide gels (SDS–15% PAGE), and Western blotting (immunoblotting) was performed with a monoclonal antibody specific for CDC2 kinase (CDC2 p34 [17]; Santa Cruz Biotechnology) and developed with the enhanced chemiluminescence assay (Amersham).

RESULTS

HIV-1 infection of SupT1 cells causes cell cycle perturbation. We analyzed cell cycle kinetics in initial experiments with HIV-1_{NL4-3} used to infect SupT1 cells at relatively high multiplicities of infection (MOIs). Analysis by hypotonic propidium iodide staining (35) and flow cytometry indicated an abnormal cell cycle profile, with a greater number of cells containing a 4*n* amount of DNA (G_2 + M) than in mock-infected cultures (Fig. 1). However, precise quantitative analysis was complicated by the presence of uninfected cells in the culture. To examine more precisely the properties of infected cells, it was necessary to distinguish infected from uninfected cells in the population in a manner that would still allow cell

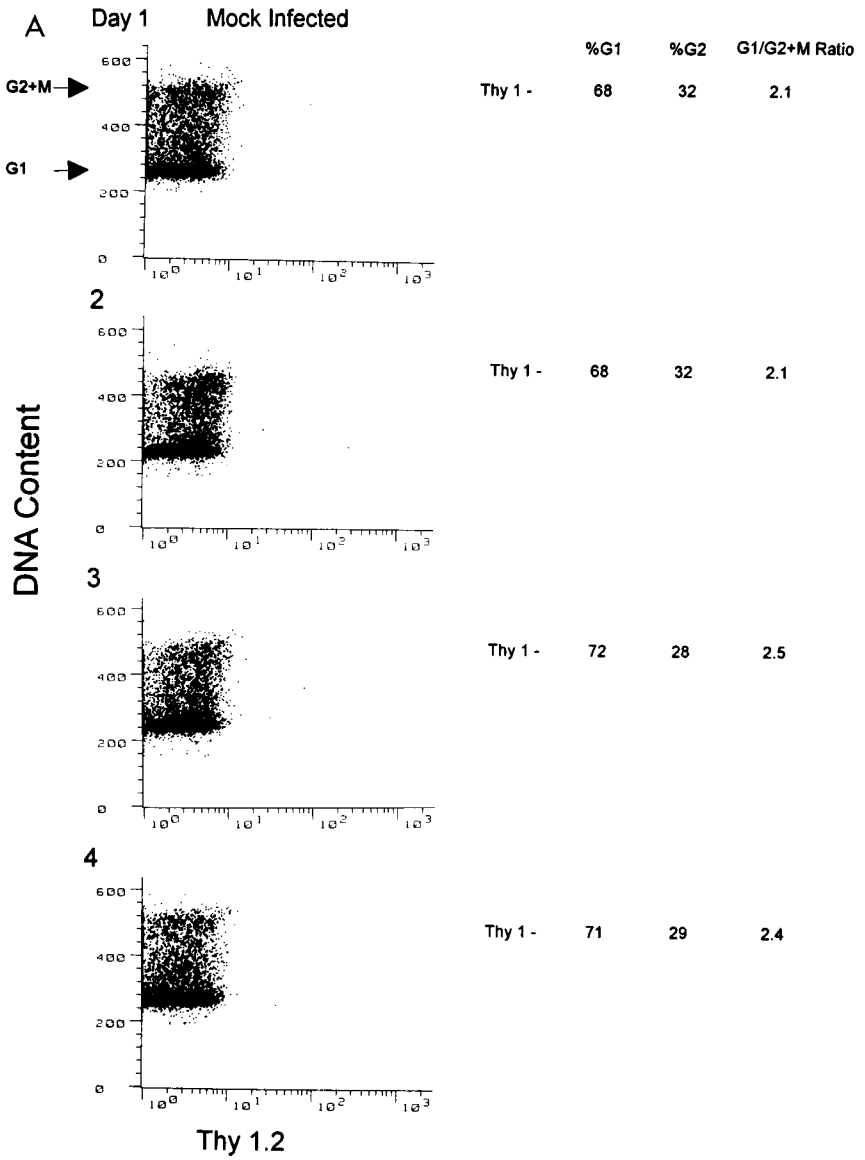


FIG. 2. Thy 1.2^+ SupT1 cells in an NL-Thy-infected culture accumulate in the G_2 + M phase of the cell cycle over time. SupT1 cells (5×10^6) were infected with NL-Thy (1.8 μg of p24). Aliquots were taken at indicated days and analyzed for cell cycle profile. (A) Mock-infected SupT1 cells were harvested at 24-h intervals postinfection, doubly stained for Thy 1.2 antigen and DNA content, and analyzed by flow cytometry. Fluorescence intensity of the propidium iodide DNA stain is plotted on the *y* axis, and Thy 1.2-FITC fluorescence intensity is plotted on the *x* axis. Arrows indicate G_1 and G_2 peaks. Percentages of cells in the G_1 and G_2 + M phases and calculated G_1/G_2 + M ratios are shown to the right of the dot plots. (B) NL-Thy-infected culture analyzed as for panel A. Percentages of cells in G_1 and G_2 + M for both Thy 1.2^- and Thy 1.2^+ subpopulations and calculated G_1/G_2 + M ratios are shown to the right of the dot plots.

cycle characterization by flow cytometry. The use of HIV-1-specific surface markers such as gp160 has been technically problematic, so we chose to use instead a replication-competent HIV-1_{NL4-3} vector (47) bearing the gene for the murine thymocyte surface antigen, *thy1.2* (13). Other marker genes have been inserted into the HIV-1 *nef* gene to generate HIV-1 vectors effective in monitoring HIV-1 infection in vitro, so we substituted *thy1.2* for a part of *nef.*

We first demonstrated that *thy1.2* expression alone did not alter cell cycle profiles by transfection of the *thy1.2* vector under the control of the CMV immediate-early promoter. The resulting cell cycle profile of the transfected Thy 1.2^+ subpopulation was identical to that of the Thy 1.2^- subpopulation and mock-transfected cells (data not shown; also see Fig. 4). The kinetics of cell cycle perturbation were determined by a time course experiment with SupT1 cells as the viral targets. After infection (MOI, ≈0.05), cells were harvested from both mock- and NL-Thy-infected cultures. A portion was stained for Thy 1.2 expression combined with a fluorescent dye to allow exclusion of dead cells, and another was doubly stained for Thy 1.2 and DNA content. On day 1 postinfection, 4% of the cells were positive for the Thy 1.2 marker; this population increased over the subsequent days to 6, 15, and 52%. Mock-infected cultures were similarly stained and found to be negative for the Thy 1.2 antigen (Fig. 2). HIV-1 core antigen (p24) levels increased over time and correlated with Thy 1.2 expression (data not shown).

Cell cycle analysis of the cultures at day 1 postinfection showed that the G_1/G_2 + M ratio was 2.3 for the Thy 1.2^- subpopulation and remained at a similar ratio for the duration

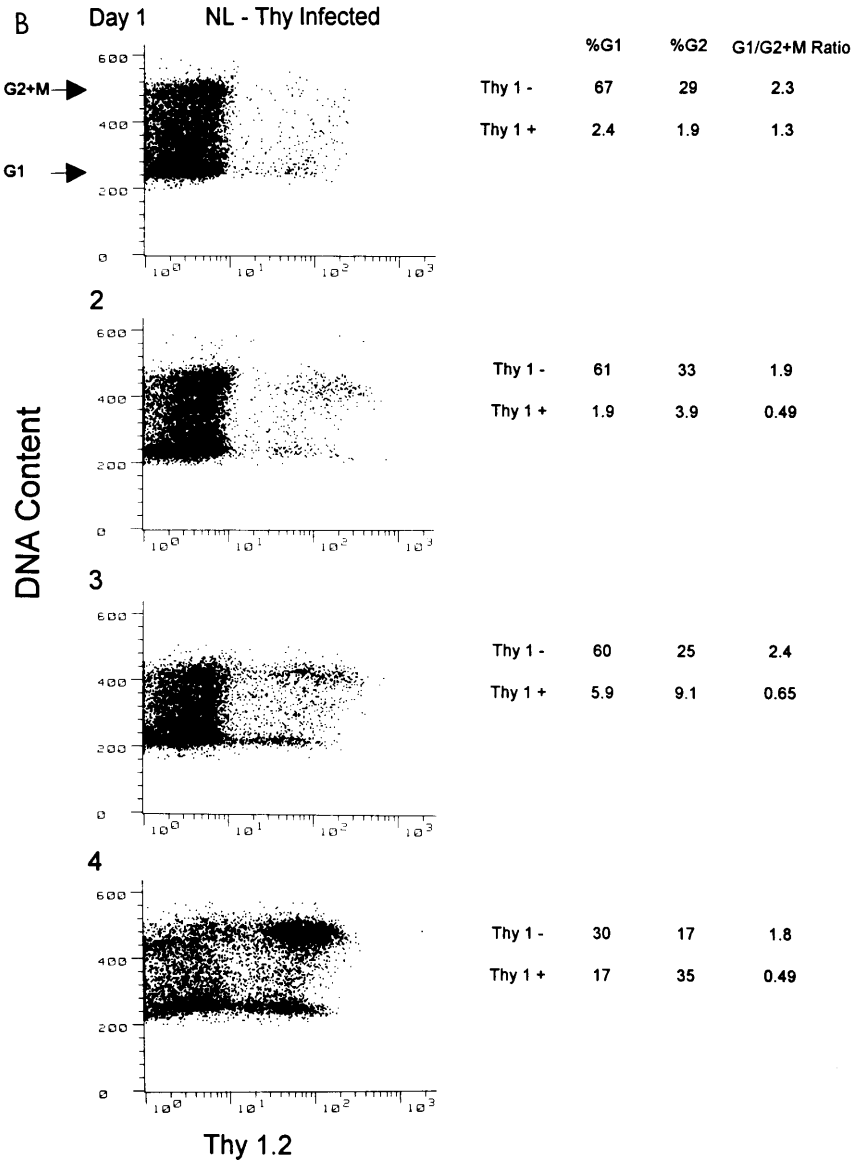


FIG. 2—*Continued.*

of the experiment (Fig. 2). Similar ratios were observed in the mock-infected culture. However, a different pattern was observed for the Thy 1.2^+ subpopulation. Initially, the G_1/G_2 + M ratio was 1.3 on day 1 postinfection and then decreased to approximately 0.5 on the following days. This indicated an accumulation of cells in G_2 + M relative to those in G_1. It was noteworthy on day 4 postinfection that although the majority of the culture was infected and cell cycle perturbation was evident, the noninfected subpopulation exhibited a normal cell cycle profile. This indicated that the uninfected cells, despite being in the same culture, were not adversely affected by the presence of the HIV-1-infected cells.

HIV-1 infection causes G_2 + M arrest in SupT1 cells. The observed inversion of the G_1/G_2 + M ratio could be explained either by the arrest and accumulation of HIV-1-infected cells in the G_2 + M phase of the cell cycle or by the death and loss of infected cells from the G_1 compartment. If the former were true, the initial number of HIV-1-infected cells in G_2 + M should stay the same or increase. However, if the increase in the proportion of G_2 + M cells were due to the death of cells in G_1, the total number of cells in G_2 + M should decrease as cells progress to G_1 and are deleted from the population. In the above experiment, these two possibilities cannot be distinguished, since the spread of HIV-1 infection through the culture can replenish the HIV-1-infected cell numbers in the G_1 and G_2 + M phases. To clarify the mechanism of the apparent G_2 + M accumulation, an experiment similar to that described above was performed, with the modification that the subsequent spread of virus within the culture was inhibited by the addition of three well-characterized HIV-1 antagonists that act at different points in the viral life cycle: AZT, which blocks reverse transcription (33); a protease inhibitor, A77003, that prevents maturation of the virion (19, 21); and sCD4, which binds to virion-associated gp120 and prevents the virion from adhering to the cell surface CD4 receptor (8, 12, 18, 40, 55, 63). The addition of sCD4 also inhibits the formation of envelope/

TABLE 1. NL-Thy-infected SupT1 cells arrest in G_2 + M[a]

Day	Thy 1.2^+ cells (10^3)			Ratio, G_1/G_2 + M
	Total	G_1	G_2 + M	
1	130	88	45	2.0
2	350	55	291	0.19
3	450	41	410	0.10
4	460	55	402	0.13

[a] SupT1 cells (5×10^6) were infected with NL-Thy (5 μg of p24), grown in the presence of sCD4, AZT, and a protease inhibitor, and analyzed by flow cytometry at indicated intervals for Thy 1.2 expression and DNA content. The actual cell numbers (percent Thy 1.2^+ times the number of cells in culture) are shown for the G_1 and G_2 + M populations, together with the calculated G_1/G_2 + M ratios.

receptor-mediated syncytia by binding to gp120 expressed on the surface of infected cells (18).

For this experiment, the absolute number of infected cells was calculated by multiplying the total cell number in the culture by the percentage of Thy 1.2^+ cells. At day 2 postinfection, the number of HIV-1-infected cells was approximately 4×10^5 and remained constant over days 3 and 4 postinfection (Table 1), although the total cell number (infected plus uninfected) increased fourfold. In the absence of inhibition, the infected cell population increased 10-fold over the same period (data not shown). The mock-infected culture was found to exhibit a cell cycle profile and growth kinetics similar to those in other experiments, demonstrating that the presence of the antagonists did not affect the growth characteristics of the cells. Likewise, analysis of the Thy 1.2^- subpopulation of the infected culture showed G_1/G_2 + M ratios similar to that of the mock-infected culture. However, the ratio for the Thy 1.2^+ subpopulation decreased from 2.0 on day 1 postinfection to approximately 0.15 from days 2 to 4 of the time course. The observations that a constant number of HIV-1-infected cells persisted in the culture over a period of 3 days and that an increasing proportion of those cells were found to be in G_2 + M over time argue strongly in favor of arrest of these cells in the G_2 + M phase of the cell cycle. As expected for the use of sCD4 in this experiment, no syncytia were observed, indicating that cytopathic effects due to Env-mediated syncytia do not play a role in the G_2 + M arrest.

HIV-1 infection causes G_2 + M arrest in stimulated PBL. We next tested whether HIV-1 could induce G_2 + M arrest in primary rather than transformed T cells. This experiment was carried out with $CD4^+$ cells enriched from PHA- and interleukin-2-stimulated PBL as targets. The proportion of Thy 1.2^+ cells increased from 2.9% on day 1 to 12% on day 4 postinfection, and the HIV-1 p24 antigen levels present in the culture supernatant also increased (Fig. 3). For PBL, the calculated proportion of cells seen by flow cytometry in the G_1 peak also includes quiescent cells in G_0. Cell cycle analysis revealed that the G_0 + G_1/G_2 + M ratio of both the mock-infected culture and the Thy 1.2^- subpopulation of the NL-Thy-infected culture remained similar throughout the time course. The ratio was observed to increase over the time period, most likely because of a relative increase in the number of T cells arresting in G_1 or reentering a quiescent G_0 state following initial activation by PHA. The Thy 1.2^+ subpopulation showed an inverted G_0 + G_1/G_2 + M ratio of 0.51 to 0.73, consistent with the data presented for infection of SupT1 cells.

Determination of viral proteins necessary for G_2 + M arrest. We determined whether the G_2 + M arrest could be attributed to specific HIV-1 genes. Since the NL-Thy virus contained a deletion in the *nef* gene, we were able to rule out the role of *nef* in G_2 + M arrest. We next tested a *vpr* insertion/frameshift mutant of the NL-Thy virus that produced a protein lacking the carboxy-terminal 33 residues of the 96-amino-acid protein. The *vpr* mutant virus, termed Vpr-X, was used to infect a culture of SupT1 cells in parallel with NL-Thy and at an equivalent MOI (approximately 0.01). Aliquots of cells were harvested from mock-, NL-Thy-, and Vpr-X-infected cultures at 24-h intervals and assayed for Thy 1.2 expression and DNA content. As expected, the Thy 1.2^- subpopulations of both the NL-Thy- and Vpr-X-infected cultures had ratios similar to that of the mock-infected culture throughout the experiment (Tables 2 and 3). Cell cycle arrest occurred in the Thy 1.2^+ subpopulation of the NL-Thy-infected culture, consistent with earlier experiments. However, the cells infected with the Vpr-X virus showed reduced cell cycle perturbation. Thus, mutation of the *vpr* gene impaired the ability of the virus to elicit G_2 + M arrest.

HIV-1 Vpr is sufficient to cause G_2 + M arrest. We demonstrated above that the *vpr* gene product contributes to induction of cell cycle arrest following infection of lymphoid cells by HIV-1. We next determined whether the *vpr* gene product was sufficient to elicit arrest in the absence of all other HIV-1 proteins. To address this question, we developed a transient-transfection and expression system to produce Vpr in target cells and examine their cell cycle profile. To allow differential analysis of transfected and nontransfected cells in the same population, a dual expression vector (called BSVprThy) was constructed; it contains the *vpr* and *thy1.2* open reading frames,

TABLE 2. NL-Thy and Vpr-X infection of SupT1 cells: cell cycle analysis

Cell and day	% of cells		Ratio, G_1/G_2 + M
	G_1	G_2	
Mock infected			
1	56	38	1.5
2	71	29	2.4
3	69	31	2.2
4	70	30	2.3
5	69	31	2.2
NL-Thy infected			
Thy 1.2^-			
1	57	38	1.5
2	71	28	2.5
3	71	26	2.7
4	62	19	3.3
5	42	35	1.2
Thy 1.2^+			
1	0.72	0.77	0.94
2	0.51	1.1	0.46
3	0.80	1.7	0.47
4	2.1	2.8	0.75
5	9.5	14	0.68
Vpr-X infected			
Thy 1.2^-			
1	62	37	1.7
2	64	34	1.9
3	65	32	2.0
4	71	25	2.8
5	56	33	1.7
Thy 1.2^+			
1	0.54	0.50	1.1
2	1.0	0.70	1.4
3	1.9	1.1	1.7
4	2.9	1.5	1.9
5	6.2	4.2	1.5

A Culture Kinetics

Day	% Thy 1+	p24 ng/ml
1	2.9	1.9
2	5.8	24
3	7.6	266
4	12	528

B Cell Cycle Analysis

Mock Infected

Day	%G1	%G2	G1/G2+M
1	65	33	2.0
2	70	28	2.5
3	77	23	3.3
4	82	18	4.6

NL-Thy Infected

Day	Thy 1 -			Thy 1 +		
	%G1	%G2	G1/G2+M	%G1	%G2	G1/G2+M
1	65	29	2.2	1.9	2.6	0.73
2	61	27	2.3	3.9	7.6	0.51
3	67	21	3.2	3.9	7.7	0.51
4	70	13	5.4	6.5	10	0.65

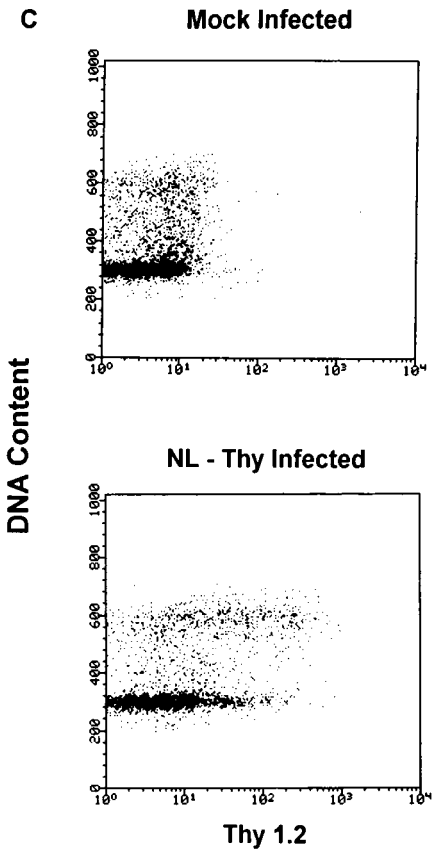


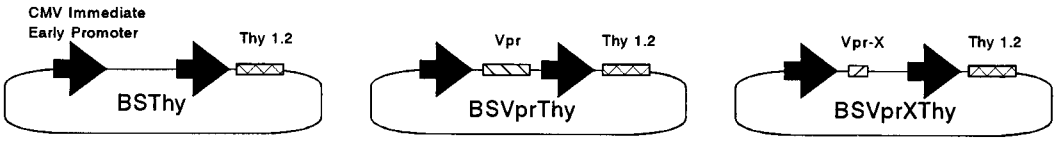
FIG. 3. NL-Thy infection of $CD4^+$ primary T lymphocytes causes cell cycle accumulation in the G_2 + M phase. PHA-stimulated primary $CD4^+$ T lymphocytes (6×10^6) were mock infected or infected with NL-Thy (1.8 μg of p24) and analyzed at 24-h intervals. (A) Analysis of infected cultures for expression of Thy 1.2 antigen and HIV-1 p24 core antigen in the culture medium. (B) Cell cycle analysis of the NL-Thy- and mock-infected cultures by double staining for Thy 1.2 antigen and DNA content. Percentages of cells in G_1 and G_2 + M and G_1/G_2 + M ratios for Thy 1.2^- and Thy 1.2^+ subpopulations are shown. (C) Dot plot analysis of mock- and NL-Thy-infected cultures at 4 days postinfection. DNA content is plotted on the *y* axis, and Thy 1.2 antigen expression is plotted on the *x* axis.

TABLE 3. NL-Thy and Vpr-X infection of SupT1 cells: culture kinetics[a]

Day	% Thy 1.2^+		p24 (ng/ml)	
	NL-Thy	Vpr-X	NL-Thy	Vpr-X
1	1.0	1.0	1.0	0.6
2	1.4	1.4	8.0	3.0
3	2.5	2.2	50	26
4	5.7	3.7	460	320
5	26	12	2,540	1,290

[a] SupT1 cells (5×10^6) were infected with NL-Thy and Vpr-X viruses (1 μg of p24) and analyzed at the indicated intervals. Infected cultures were analyzed for expression of Thy 1.2 antigen and HIV-1 p24 core antigen in the culture medium.

each under the control of a separate CMV immediate-early promoter (Fig. 4). As a control, an otherwise identical expression plasmid lacking the *vpr* open reading frame was also constructed (BSThy). The control vector had no effect on cell cycle profiles. Furthermore, a dual expression vector containing the same mutant *vpr* open reading frame as that present in the Vpr-X virus did not cause cell cycle perturbation (Fig. 4, experiments 2 and 3). The Thy 1.2^+ subpopulation of the Vpr-containing BSVprThy-transfected culture showed an inverted ratio of 0.67, consistent with that seen in virus-infected cells, and the Thy 1.2^- subpopulation was normal. We therefore conclude that expression of the HIV-1 *vpr* gene product alone is sufficient to cause G_2 + M arrest. Cell cycle arrest was also observed in COS, CV-1 (African green monkey kidney), and HeLa cells (human cervical carcinoma), but not in NIH 3T3 (murine embryonic) cells (data not shown).



Cell Line	BSThy		BSVprThy		BSVprXThy	
Expt. 1	G1/G2+M Ratio		G1/G2+M Ratio		G1/G2+M Ratio	
	Thy 1.2 −	Thy 1.2 +	Thy 1.2 −	Thy 1.2 +	Thy 1.2 −	Thy 1.2 +
SupT1	1.3	1.3	1.2	0.67	ND	ND
HeLa	1.6	2.3	1.7	0.32	ND	ND
COS	2.2	1.5	2.2	0.54	ND	ND
Expt. 2						
HeLa	ND	ND	1.6	0.28	2.0	2.0
Expt. 3						
CV-1	ND	ND	2.8	0.70	4.2	3.8

FIG. 4. HIV-1 Vpr protein causes G_2 + M arrest in various cell types. In experiment 1, SupT1 (5×10^6), HeLa (10^7), and COS (10^7) cells were transfected with BSVprThy (10 μg), a plasmid that expresses both the Thy 1.2 antigen and the HIV-1 Vpr protein, and a control plasmid (10 μg), BSThy, that expresses Thy 1.2 alone. Cells were harvested at 48 h posttransfection and doubly stained for Thy 1.2 expression and DNA content. In two additional experiments (2 and 3), HeLa and CV-1 cells were transfected with BSVprThy (10 μg) and another plasmid carrying a mutated *vpr* open reading frame, BSVprXThy (10 μg), and analyzed as for experiment 1. These plasmids also contained an untranslated CMV intron, as described in Materials and Methods. Transfection efficiencies monitored by Thy 1.2 expression were as follows: experiment 1, SupT1 cells, 3%; HeLa cells, 45%; and COS cells, 19%; experiment 2, HeLa cells, 50%; and experiment 3, CV-1 cells, 10%. (Top) Schematic structure of plasmids. The bold arrow indicates the CMV immediate-early promoter, the hatched bar indicates the position of *thy1.2*, and the long and short shaded bars indicate the *vpr* and *vpr-X* genes, respectively. (Bottom) The ratios of cells in G_1 to cells in G_2 + M for the Thy 1.2^- and Thy 1.2^+ populations are given. ND, not done.

Alteration of CDC2 kinase. One of the key regulatory factors involved in progression of cells from the G_2 phase into M is the serine/threonine kinase CDC2. It has previously been demonstrated that dephosphorylation of this kinase at two critical residues, Thr-14 and Tyr-15, is necessary for activation of CDC2 kinase (23, 57, 58) and subsequent phosphorylation events mediated by CDC2 that lead to progression into mitosis. Chemical agents that block DNA synthesis or that induce DNA damage can arrest cells in G_2 through activation of pathways that lead to an accumulation of hyperphosphorylated CDC2 kinase (34, 41). The hyperphosphorylated form of CDC2 kinase has previously been shown to be distinguishable from other forms by decreased mobility on gels (9, 37). Therefore, we tested by SDS-PAGE whether cells arrested by Vpr show evidence of alterations in the phosphorylation state of CDC2 kinase. Two different Vpr-expressing constructs, BSVpr and BSVprThy, showed increased levels of the hyperphosphorylated form of CDC2 relative to a control transfection with a vector expressing the *cdc2* gene (Fig. 5A). In a second experiment, we used nocodazole and nitrogen mustard (HN_2) as controls for different forms of CDC2. Nocodazole is an inhibitor of mitotic spindle formation and arrests cells in mitosis, when the majority of the CDC2 is in an active nonhyperphosphorylated form (20, 37, 41). Consistent with previously published reports, nocodazole-treated cells contained little or no hyperphosphorylated CDC2 (Fig. 5B, Noc). Nitrogen mustard, in contrast, induces DNA damage, which delays cells in G_2, resulting in greater levels of hyperphosphorylated CDC2 (41). As expected, nitrogen mustard-treated cells showed greater levels of the hyperphosphorylated form of CDC2 (Fig. 5B, HN_2). Cells transfected with Vpr-expressing constructs showed increased levels of the hyperphosphorylated form of CDC2 kinase, whereas those transfected with Vpr-X-containing vectors did not (Fig. 5B, BSVprThy and BSVprXThy). Thus, these results are consistent with the known properties of CDC2 kinase in cells blocked at the G_2/M transition and provide further support for our conclusion that Vpr acts to block cells in the G_2 + M phase of the cell cycle.

DISCUSSION

Here we report that HIV-1 infection of CD4 T lymphocytes and T lymphoblastoid cell lines causes cell cycle perturbation, resulting in an apparent accumulation of cells in the second gap (G_2) or mitosis (M) phase of the cell cycle in an asynchronously dividing population of cells. With a marked retrovirus vector and by inhibiting the spread of the virus through the culture, it was apparent that cells infected with HIV-1 were unable to transit normally from the G_2 phase through mitosis to complete the cell cycle, thus demonstrating G_2 + M arrest as opposed to G_2 + M accumulation due to death of infected cells in G_1. Cell cycle arrest in the G_2 + M phase occurred in the absence of syncytium formation or other visible cytopathic effect. The cause of cell cycle arrest was attributed to the HIV-1 *vpr* gene product. Mutant viruses lacking the full-length *vpr* open reading frame failed to inhibit cell cycle progression.

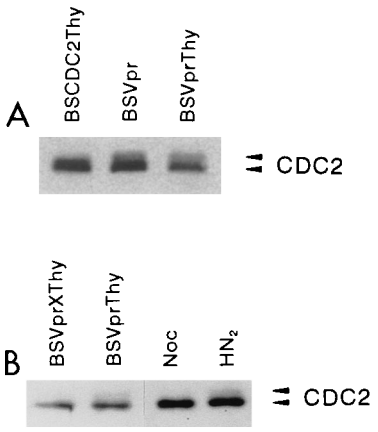


FIG. 5. HIV-1 Vpr protein induces increased levels of the hyperphosphorylated form of CDC2 kinase. HeLa cells were lysed at 48 h posttransfection and analyzed by Western blotting with a CDC2-specific monoclonal antibody. The hyperphosphorylated form of CDC2 kinase is detected as a more slowly migrating form of CDC2 (9, 37, 41). (A) HeLa cells (10^7) were transfected, as described in Materials and Methods, with BSCDC2Thy, a plasmid that expresses both human CDC2 and the Thy 1.2 antigen, or with expression plasmids that express Vpr alone (BSVpr) or both Vpr and Thy 1.2 antigen (BSVprThy). In this experiment, the expression vectors for Vpr also contained a nonapeptide epitope derived from the influenza virus hemagglutination protein at the amino terminus of Vpr, which does not affect the ability of Vpr to cause G_2 + M arrest in HeLa cells (data not shown). Ten micrograms of total protein was loaded per lane. (B) HeLa cells (10^7) were transfected with BSVprThy or BSVprXThy, as described in the legend to Fig. 4. Transfection efficiencies monitored by Thy 1.2 expression were 40% for BSVprThy and 53% for BSVprXThy. G_1/G_2 + M ratios for BSVprThy-transfected cells were 2.2 for the Thy 1.2^- population and 0.5 for the Thy 1.2^+ population; for BSVprXThy-transfected cells, they were 1.5 and 2.3 for the Thy 1.2^- and Thy 1.2^+ populations, respectively. Nocodazole (Noc)-treated HeLa cells were incubated with 0.5 μg of nocodazole per ml in the medium for 12 h before harvesting. HeLa cells were treated with nitrogen mustard (HN_2) at 0.5 μM for 30 min, resuspended in medium without HN_2, and analyzed 12 h later. The G_1/G_2 + M ratios for untreated and nocodazole-, and HN_2-treated cells were 2.9, 0.02, and 1.0, respectively. Five micrograms of total protein was loaded per lane.

Transient transfection of *vpr* expression constructs in cells revealed that expression of Vpr alone was sufficient to elicit cell cycle arrest. Cell cycle arrest correlates with an increase in the level of hyperphosphorylated CDC2, indicating an arrest of cells at the G_2/M boundary. The inhibition of cellular proliferation in a number of cell types, including primary T cells, suggests a novel mechanism for HIV-1-induced immune dysfunction.

Recent studies in our laboratory (46) and others (48) have identified a role for HIV-1 Vpr in preventing the establishment of chronic viral infection of the host cell. Those results suggested that Vpr altered the growth properties of HIV-1-infected cells in a population by either causing the death of cells or arresting cell proliferation. Rogel et al. (48) further demonstrated that the cell cycle profiles of cells transfected with Vpr were altered, leading to a greater proportion of cells in the G_2 or M phase. However, those studies did not distinguish between cytolytic and cytostatic mechanisms. The data presented here are concordant with these findings and expand on them by directly demonstrating that cell cycle arrest occurs in HIV-1-infected T cells. We also established that the cell cycle arrest of infected cells occurs in the G_2 or M phase of the cell cycle.

Vpr is known to be incorporated into the virion via a direct interaction with the HIV-1 Gag protein (6, 22, 26, 45, 65). Its presence in the virion particle facilitates nuclear localization of viral nucleic acids following entry in nondividing monocyte-derived macrophages and mimosine growth-arrested lymphoid cell lines (17). In support of the latter hypothesis, Vpr was found to be required for efficient replication of HIV in human macrophages but not PBL or an immortalized T-cell line (3, 16).

Transfection of Vpr has been observed to promote differentiation and growth inhibition of a human rhabdomyosarcoma cell line previously used to study terminal differentiation and tumorigenesis of muscle cells (28). Although the cause and effect of growth arrest versus differentiation was not established, the observed inhibition of growth is consistent with our data showing G_2 + M arrest in multiple cell types. These include COS, CV-1, HeLa, and SupT1 cells; however, G_2 + M arrest was not observed in the murine cell line NIH 3T3. Other experiments show similar arrest in MT-2 and HUT 78 cell lines (20a).

Vpr has also been described as a weak transcriptional activator and can increase both the viral replication rate and cytopathic effect of HIV-1 in vitro (7, 42). We noted that there was an approximately threefold increase in surface levels of Thy 1.2 antigen on infected cells in G_2 + M compared with that on those in G_1 (Fig. 2) and that cultures infected with virus capable of producing Vpr yielded higher levels of p24 core antigen (Table 3). It is possible that Vpr exerts a *trans*-activating effect in the G_2 + M phase of the cell cycle. Alternatively, the G_2 + M phase of the cell cycle may be more conducive to the function of certain promoters, including the HIV-1 LTR.

Vpr is likely to act upon one or more of the steps necessary for transition through mitosis into G_1. One critical event that drives mammalian cells from G_2 into mitosis is the activation of the maturation-promoting factor, whose components include CDC2 kinase and cyclin B. The CDC2 kinase is highly regulated, requiring dephosphorylation by CDC25 for its activation. Antagonizing this reaction is the Wee 1 kinase, which phosphorylates CDC2 and thus inhibits the transition into mitosis (36, 38, 39, 57). Additional control elements may regulate these enzymes; for example, the Nim 1 kinase has been shown to inhibit the Wee 1 homolog in fission yeast (44), although a mammalian counterpart has not yet been described. Our results show an increase in the inactive hyperphosphorylated form of the CDC2 kinase. This suggests that the Vpr protein acts to antagonize the activation of CDC2 kinase either directly or indirectly through modulation of its regulatory cascades. By analogy with other factors, such as DNA-damaging agents that are known to block the progression of cells from G_2 to M through inhibition of the activation of CDC2 kinase (41), detection of increased levels of hyperphosphorylated CDC2 kinase in our experiments suggests but does not prove that the cell cycle arrest is more likely in late G_2 phase than in mitosis.

We consider a number of possibilities for the role that G_2 + M arrest plays in HIV-1 replication. First, an activated T cell can differentiate into a G_0-phase memory cell, having passed through mitosis (59, 60). Such a cell would exhibit minimal metabolic and transcriptional activity and, as such, be unable to efficiently produce viral proteins. An activated T cell harboring HIV-1 and arrested in the G_2 + M phase of the cell cycle would be prevented from continuing the cell cycle and potentially reentering G_0. Thus, by prolonging the time that a host cell spends in the activated state, the virus may maximize the output of progeny virus. Second, in addition to reentering G_0, an activated T cell can undergo apoptosis; G_2 + M arrest may prevent this. A recent report indicated that activation of the CDC2 kinase was required for certain cell types to undergo apoptosis (54). Our results indicated that HIV-1 Vpr could prevent or delay the host cell from initiating apoptosis through inhibition of this kinase. It has been suggested, however, that HIV-1 can kill cells by apoptosis (14, 25, 61). These hypotheses

may not necessarily be incompatible. HIV-1-infected immortalized SupT1 cells were observed in our experiments to remain in the G_2 + M state for at least 3 days, approximately three times longer than the natural duration of the typical mammalian cell cycle. The subsequent fate of these cells is unknown, and it is possible that they eventually die by apoptosis. Third, as indicated above, we have observed that NL-Thy-infected cells in G_2 + M express threefold more Thy 1.2 surface antigen than those in G_1. This suggests that cells in G_2 + M may be more efficient in producing proteins under the control of the HIV-1 LTR. The observation that Vpr can act as a weak transcriptional activator (7) may be related to this phenomenon, although the mechanism is not clear. Cell cycle-dependent protein production has been well documented for the cyclins, a class of proteins that regulate various events in cell cycle progression (38). HIV-1 LTR promoter responsiveness to cell cycle phase has not been reported; however, it is possible that the availability of transcription factors known to interact with the promoter fluctuates during cell replication. For example, it has been reported that the cell surface replacement rate of CD4 is highest during late DNA synthesis phase (S) and in G_2 (31). Fourth, another advantage of inhibition of T-cell proliferation for HIV-1 is in directly limiting the extent and breadth of the immune response directed against it (see below).

The implications of Vpr-induced G_2 + M arrest on the immunopathogenesis of AIDS are potentially significant. In the lymphoid organs, a successful immune response depends on activation by antigen and subsequent clonal expansion and migration of antigen-specific T cells into the circulation. We propose here a novel mechanism for immune dysfunction, in which an activated T-cell population could be infected with HIV-1 and, as a consequence, be unable to proliferate further, thus aborting clonal expansion. Limiting this critical phase of the cellular immune response would likely result in additional disruption of downstream events dependent on the clonal expansion of the CD4 T-lymphocyte subpopulation. Such events may include T-cell helper activity via release of cytokines, stimulation and modulation of the humoral immune response, differentiation resulting in the generation of memory cells, and $CD4^+$ cytotoxic T-cell activity.

The targeting of the Vpr protein or its mode of action may offer a new avenue for therapeutic intervention in the pathogenesis of AIDS. Notably, in an animal model system, mutation of the *vpr* open reading frame from the related retrovirus simian immunodeficiency virus (SIV) dramatically reduced virus load and persistence in macaques compared with wild-type SIV (24). Monkeys in which the *vpr* mutation did not revert showed no signs of disease development during the observation period. Therapy directed against Vpr function may offer a novel twofold approach for ameliorating disease progression. In the first instance, blocking Vpr action would likely reduce the efficiency of viral replication. However, as with other virally targeted drugs, the development of resistance is cause for concern. Second, blocking Vpr-mediated cell cycle arrest would allow proliferation of infected cells and would therefore be likely to augment the immune response. A putative therapeutic operating by this latter mechanism would be particularly attractive, as it could circumvent the problem of virally acquired drug resistance by acting on cellular components to overcome the block to cell cycle progression.

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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., and A. Capron.** 1991. Cell dysfunction and depletion in AIDS: the programmed cell death hypothesis. Immunol. Today **12:**102–105.
3. **Balotta, C., P. Lusso, R. Crowley, R. C. Gallo, and G. Franchini.** 1993. Antisense phosphorothioate oligodeoxynucleotides targeted to the *vpr* gene inhibit human immunodeficiency virus type 1 replication in primary human macrophages. J. Virol. **67:**4409–4414.
4. **Banda, N. K., J. Berrier, 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.
5. **Cheng-Mayer, C., D. Seto, M. Tateno, and J. A. Levy.** 1988. Biologic features of HIV-1 that correlate with virulence in the host. Science **240:**80–82.
6. **Cohen, E. A., G. Dehni, J. G. Sodroski, and W. A. Haseltine.** 1990. Human immunodeficiency virus *vpr* product is a virion-associated regulatory protein. J. Virol. **64:**3097–3099.
7. **Cohen, E. A., E. F. Terwilliger, Y. Jalinoos, J. Proulx, J. G. Sodroski, and W. A. Haseltine.** 1990. Identification of HIV-1 vpr product and function. J. Acquired Immune Defic. Syndr. **3:**11–18.
8. **Deen, K. C., J. S. McDougal, R. Inacker, G. Golena-Wasserman, J. Arthos, J. Rosenberg, P. J. Maddon, R. Axel, and R. W. Sweet.** 1988. A soluble form of CD4 (T4) protein inhibits AIDS virus infection. Nature (London) **331:** 82–84.
9. **Draetta, G., and D. Beach.** 1988. Activation of *cdc*2 protein kinase during mitosis in human cells: cell-cycle-dependent phosphorylation and subunit rearrangement. Cell **54:**17–26.
10. **Fauci, A. S.** 1993. Multifactorial nature of human immunodeficiency virus disease: implications for therapy. Science **262:**1011–1018.
11. **Fauci, A. S.** 1988. The human immunodeficiency virus: infectivity and mechanisms of pathogenesis. Science **239:**617–622.
12. **Fisher, R. A., J. M. Bertonis, W. Meier, V. A. Johnson, D. S. Costopoulos, T. Liu, R. Tizard, B. D. Walker, M. S. Hirsh, R. T. Schooley, and R. A. Flavell.** 1988. HIV infection is blocked *in vitro* by recombinant soluble CD4. Nature (London) **331:**76–78.
13. **Giguere, V., K. I. Isobe, and F. Grosveld.** 1985. Structure of the murine Thy-1 gene. EMBO J. **4:**2017–2024.
14. **Gougeon, M. L., A. G. Laurent-Crawford, A. Hovanessian, and L. Montagnier.** 1993. Direct and indirect mechanisms mediating apoptosis during HIV infection: contribution to in vivo CD4 T cell depletion. Semin. Immunol. **5:**187–194.
15. **Groux, H., G. Torpier, D. Monte, Y. Mouton, A. Capron, and J. C. Ameison.** 1992. Activation-induced death by apoptosis in $CD4^+$ T cells from human immunodeficiency virus-infected asymptomatic individuals. J. Exp. Med. **175:**331–340.
16. **Hattori, N., F. Michaels, K. Fargnoli, L. Marcon, R. C. Gallo, and G. Franchini.** 1990. The human immunodeficiency virus type 2 vpr gene is essential for productive infection of human macrophages. Proc. Natl. Acad. Sci. USA **87:**8080–8084.
17. **Heinzinger, N. K., M. I. Bukrinsky, S. A. Haggerty, A. M. Ragland, V. Kewalramani, M.-A. Lee, H. E. Gendelman, L. Ratner, M. Stevenson, and M. Emerman.** 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. Proc. Natl. Acad. Sci. USA **91:**7311–7315.
18. **Hussey, R. E., N. E. Richardson, M. Kowalski, N. R. Brown, H.-C. Chang, R. F. Siliciano, T. Dorfman, B. Walker, J. Sodroski, and E. L. Reinhartz.** 1988. A soluble CD4 protein selectively inhibits HIV replication and syncytium formation. Nature (London) **331:**78–81.
19. **Jaskolski, M., A. G. Tamasselli, T. K. Sawyer, D. G. Staples, R. L. Heinrikson, J. Schneider, S. B. Kent, and A. Wlodawer.** 1991. Structure at 2.5-Å resolution of chemically synthesized human immunodeficiency virus type 1 protease complexed with a hydroxyethylene-based inhibitor. Biochemistry **30:**1600–1609.
20. **Jordan, M. A., D. Thrower, and L. Wilson.** 1992. Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles: implications for the role of microtubule dynamics in mitosis. J. Cell Sci. **102:**401–416.

20a. **Jowett, J. B. M., and V. Planelles.** Unpublished data.

21. **Kaplan, A. H., J. A. Zack, M. Knigge, D. A. Paul, D. J. Kempf, D. W. Norbeck, and R. Swanstrom.** 1993. Partial inhibition of human immunode-

ficiency virus type 1 protease results in aberrant virus assembly and the formation of noninfectious particles. J. Virol. **67:**4050–4055.
22. **Kondo, E., F. Mammano, E. A. Cohen, and H. G. Gottlinger.** 1995. The p6^{gag} domain of human immunodeficiency virus type 1 is sufficient for the incorporation of Vpr into heterologous viral particles. J. Virol. **69:**2759–2764.
23. **Krek, W., and E. A. Nigg.** 1991. Differential phosphorylation of vertebrate p34^{cdc2} kinase at the G1/S and G2/M transitions of the cell cycle: identification of major phosphorylation sites. EMBO J. **10:**305–316.
24. **Lang, S. M., M. Weeger, C. Stahl-Hennig, C. Coulibaly, G. Hunsmann, J. Muller, H. Muller-Hermelink, D. Fuchs, H. Wachter, M. M. Daniel, R. C. Desrosiers, and B. Fleckenstein.** 1993. Importance of *vpr* for infection of rhesus monkeys with simian immunodeficiency virus. J. Virol. **67:**902–912.
25. **Laurent-Crawford, A. G., B. Drust, 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. **Lavallee, C., X. J. Yao, A. Ladha, H. Gottlinger, W. A. Haseltine, and E. A. Cohen.** 1994. Requirement of the Pr55^{gag} precursor for incorporation of the Vpr product into human immunodeficiency virus type 1 viral particles. J. Virol. **68:**1926–1934.
27. **Lee, M. G., and P. Nurse.** 1987. Complementation used to clone a human homologue of the fission yeast cell cycle control gene *cdc2*. Nature (London) **327:**31–35.
28. **Levy, D. N., L. S. Fernandes, W. V. Williams, and D. B. Weiner.** 1993. Induction of cell differentiation by human immunodeficiency virus 1 vpr. Cell **72:**541–550.
29. **Lifson, J. D., M. B. Feinberg, G. R. Reyes, L. Rabin, B. Banapour, S. Chakrabarti, B. Moss, F. Wong-Staal, K. S. Steimer, and E. G. Engleman.** 1986. Induction of CD4-dependent cell fusion by the HTLV-III/LAV envelope glycoprotein. Nature (London) **323:**725–728.
30. **Lifson, J. D., G. R. Reyes, M. S. McGrath, B. S. Stein, and E. G. Engleman.** 1986. AIDS retrovirus induced cytopathology: giant cell formation and involvement of CD4 antigen. Science **232:**1123–1127.
31. **Martin, C. F., J. Correale, S. Kam-Hansen, and A. Ehrnst.** 1992. Cell cycle-dependent expression of CD4 antigen in a monocytoid cell line. Scand. J. Immunol. **34:**483–489.
32. **Meyaard, L., S. A. Otto, R. R. Jonker, M. J. Mijnster, R. P. Keet, and F. Miedema.** 1992. Programmed death of T cells in HIV-1 infection. Science **257:**217–219.
33. **Mitsuya, H., K. J. Weinhold, P. A. Furman, M. H. St. Clair, S. N. Lehrman, R. C. Gallo, D. Bolognesi, D. W. Barry, and S. Broder.** 1985. 3′-Azido-3′-deoxythymidine (BW A509U): an antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphadenopathy-associated virus *in vitro*. Proc. Natl. Acad. Sci. USA **82:**7096–7100.
34. **Murray, A. W.** 1992. Creative blocks: cell-cycle checkpoints and feedback controls. Nature (London) **359:**599–604.
35. **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.
36. **Nigg, E. A.** 1993. Targets of cyclin-dependent protein kinases. Curr. Opin. Cell Biol. **5:**187–193.
37. **Norbury, C., J. Blow, and P. Nurse.** 1991. Regulatory phosphorylation of the p34^{cdc2} protein kinase in vertebrates. EMBO J. **10:**3321–3329.
38. **Norbury, C., and P. Nurse.** 1992. Animal cell cycles and their control. Annu. Rev. Biochem. **61:**441–470.
39. **Nurse, P.** 1990. Universal control mechanism regulating onset of M-phase. Nature (London) **344:**503–508.
40. **O'Brien, W. A., E. S. Daar, D. D. Ho, and I. S. Y. Chen.** 1992. Mapping genetic determinants for human immunodeficiency virus type 1 resistance to soluble CD4. J. Virol. **66:**3125–3130.
41. **O'Connor, P. M., D. K. Ferris, M. Pagano, G. Draetta, J. Pines, T. Hunter, D. L. Longo, and K. W. Kohn.** 1993. G_2 delay induced by nitrogen mustard in human cells affects cyclin A/cdk2 and cyclin B1/cdc2-kinase complexes differently. J. Biol. Chem. **268:**8298–8308.
42. **Ogawa, K., R. Shibata, T. Kiyomasu, I. Higuchi, Y. Kishida, A. Ishimoto, and A. Adachi.** 1989. Mutational analysis of the human immunodeficiency virus *vpr* open reading frame. J. Virol. **63:**4110–4114.
43. **Pantaleo, G., C. Graziosi, and A. S. Fauci.** 1993. New concepts in the immunopathogenesis of human immunodeficiency. N. Engl. J. Med. **328:** 327–335.
44. **Parker, L. L., S. A. Walter, P. G. Young, and H. Piwnica-Worms.** 1993. Phosphorylation and inactivation of the mitotic inhibitor Wee 1 by the nim1/cdr1 kinase. Nature (London) **363:**736–738.
45. **Paxton, W., R. I. Connor, and N. R. Landau.** 1993. Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of *gag* and mutational analysis. J. Virol. **67:**7229–7237.
46. **Planelles, V., F. Bachelerie, J. B. M. Jowett, A. Haislip, Y. Xie, P. Banooni, T. Masuda, and I. S. Y. Chen.** Unpublished data.
47. **Planelles, V., A. Haislip, E. S. Withers-Ward, S. A. Stewart, Y. Xie, N. P. Shah, and I. S. Y. Chen.** 1995. A new reporter system for detection of viral infection. Gene Ther. **2:**369–376.
48. **Rogel, M. E., L. I. Wu, and M. Emerman.** 1995. The human immunodeficiency virus type 1 *vpr* gene prevents cell proliferation during chronic infection. J. Virol. **69:**882–888.
49. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
50. **Schmid, I., C. H. Uittenbogaart, and J. V. Giorgi.** 1991. A gentle fixation and permeabilization method for combined cell surface and intracellular staining with improved precision in DNA quantification. Cytometry **12:**279–285.
51. **Shaw, G. M., B. H. Hahn, S. K. Arya, J. E. Groopman, R. C. Gallo, and F. Wong-Staal.** 1984. Molecular characterization of human T-cell leukemia (lymphotropic) virus type III in the acquired immune deficiency syndrome. Science **226:**1165–1170.
52. **Shearer, G. M., and M. Clerici.** 1993. How human immunodeficiency virus ravages the immune system. Curr. Opin. Immunol. **4:**463–465.
53. **Sheppard, H. W., and M. S. Ascher.** 1992. The natural history and pathogenesis of HIV infection. Annu. Rev. Microbiol. **46:**533–564.
54. **Shi, L., W. K. Nishioka, J. Th'ng, E. M. Bradbury, D. W. Litchfield, and A. H. Greenberg.** 1994. Premature p34^{cdc2} activation required for apoptosis. Science **263:**1143–1145.
55. **Smith, D. H., R. A. Byrn, S. A. Marsters, T. Gregory, J. E. Groopman, and D. J. Capon.** 1987. Blocking of HIV-1 infectivity by a soluble, secreted form of the CD4 antigen. Science **238:**1704–1707.
56. **Sodroski, J., W. C. Goh, C. Rosen, K. Campbell, and W. Haseltine.** 1986. Role of the HTLV-III/LAV envelope in syncytium formation and cytopathogenicity. Nature (London) **322:**470–474.
57. **Solomon, M. J.** 1993. Activation of the various cyclin/cdc2 protein kinases. Curr. Opin. Cell Biol. **5:**180–186.
58. **Solomon, M. J., M. Glotzer, T. H. Lee, M. Philippe, and M. W. Kirschner.** 1990. Cyclin activation of p34^{cdc2}. Cell **63:**1013–1024.
59. **Sprent, J.** 1994. T and B memory cells. Cell **76:**315–322.
60. **Sprent, J., and D. F. Tough.** 1994. Lymphocyte life-span and memory. Science **265:**1395–1400.
61. **Terai, C., R. S. Kornbluth, C. D. Pauza, D. D. Richmann, and D. A. Carson.** 1991. Apoptosis as a mechanism of cell death in cultured lymphoblasts acutely infected with HIV-1. J. Clin. Invest. **87:**1710–1715.
62. **Tersmette, M., R. A. Gruters, F. de Wolf, R. E. Y. de Goede, J. M. A. Lange, P. T. A. Schellekens, J. Goudsmit, H. G. Huisman, and F. Miedema.** 1989. Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of acquired immunodeficiency syndrome: studies on sequential HIV isolates. J. Virol. **63:**2118–2125.
63. **Traunecker, A., W. Luke, and K. Karjalainen.** 1988. Soluble CD4 molecules neutralize human immunodeficiency virus type I. Nature (London) **331:**84–86.
64. **Wysocki, L. J., and V. L. Sato.** 1978. "Panning" for lymphocytes: a method for cell selection. Proc. Natl. Acad. Sci. USA **75:**2844–2848.
65. **Yuan, X., Z. Matsuda, M. Matsuda, M. Essex, and T. H. Lee.** 1990. Human immunodeficiency virus vpr gene encodes a virion-associated protein. AIDS Res. Hum. Retroviruses **6:**1265–1271.
66. **Zagury, D., J. Bernard, R. Leonard, R. Cheynier, M. Feldman, P. S. Sarin, and R. C. Gallo.** 1986. Long term cultures of HTLV-III-infected cells: a model of cytopathology of T cell depletion in AIDS. Science **231:**850–853.