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Extracellular Vpr Protein Increases Cellular Permissiveness to Human Immunodeficiency Virus Replication and Reactivates Virus from Latency

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The *vpr* gene product of human immunodeficiency virus (HIV) and simian immunodeficiency virus is a virion-associated regulatory protein that has been shown using *vpr* mutant viruses to increase virus replication, particularly in monocytes/macrophages. We have previously shown that *vpr* can directly inhibit cell proliferation and induce cell differentiation, events linked to the control of HIV replication, and also that the replication of a *vpr* mutant but not that of wild-type HIV type 1 (HIV-1) was compatible with cellular proliferation (D. N. Levy, L. S. Fernandes, W. V. Williams, and D. B. Weiner, Cell 72:541–550, 1993). Here we show that purified recombinant Vpr protein, in concentrations of <100 pg/ml to 100 ng/ml, increases wild-type HIV-1 replication in newly infected transformed cell lines via a long-lasting increase in cellular permissiveness to HIV replication. The activity of extracellular Vpr protein could be completely inhibited by anti-Vpr antibodies. Extracellular Vpr also induced efficient HIV-1 replication in newly infected resting peripheral blood mononuclear cells. Extracellular Vpr transcomplemented a *vpr* mutant virus which was deficient in replication in promonocytic cells, restoring full replication competence. In addition, extracellular Vpr reactivated HIV-1 expression in five latently infected cell lines of T-cell, B-cell, and promonocytic origin which normally express very low levels of HIV RNA and protein, indicating an activation of translational or pretranslational events in the virus life cycle. Together, these results describe a novel pathway governing HIV replication and a potential target for the development of anti-HIV therapeutics.

Human immunodeficiency virus (HIV) replication is controlled at multiple levels. The interplay of extracellular factors (e.g., cytokines, hormones, activated immune cells, and other pathogens) and intracellular factors (e.g., transcription factors and DNA replication factors) and viral regulatory proteins establish positive and negative regulatory systems controlling virus expression (for reviews, see references 13, 31, 40, 50, 62). The establishment of either productive or nonproductive (latent) HIV infection is regulated both by the virus and by the status of the host cells through poorly defined mechanisms. Latency in HIV infection may be an important mechanism for viral persistence in vivo. The permissiveness of target cells to infection and replication is linked to cellular proliferation, activation, and differentiation, which are in turn regulated by a variety of host factors. For example, T-cell activation is required for the completion of viral reverse transcription and integration (5, 60, 69), though DNA synthesis and cell division are not required for HIV replication in T cells (34, 35). Infected resting T cells may provide a pool of latently infected cells from which virus may be induced after immune stimulation in vivo (3, 70). On the other hand, HIV and lentiviruses in general are unusual retroviruses in their ability to replicate in nonproliferating terminally differentiated macrophages (reviewed in reference 13). Macrophages constitute a second major reservoir for HIV (20, 25, 33, 43) and are often latently infected in vivo (12, 41), and virus replication can be induced by immune activation (41, 54, 55).

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The *vpr* open reading frame of HIV type 1 (HIV-1) encodes a 96-amino-acid protein with an apparent molecular size of 12 to 15 kDa (66). In vitro studies using *vpr* mutant viruses indicated somewhat slower replication kinetics than those with wild-type virus in T-lymphoid lines and primary T cells (44, 56, 57) and particularly poor replication in primary monocytes (24). Antisense *vpr* phosphorothioate deoxynucleotides have been reported to inhibit HIV-1 replication in macrophages (1). A *vpr-nef* mutant simian immunodeficiency virus (SIV) failed to establish pathogenic infection in monkeys (28), suggesting a vital role in replication in vivo. However the mechanism through which Vpr acts is not known. Vpr may assist Gag functions (37, 47), nuclear localization of the preintegration complex (71), and/or it may assist HIV transcription (7). We have previously reported that *vpr*, alone or in the context of the virus, arrests the proliferation of and induces gross morphological changes in a variety of cell types and allows differentiation of muscle tumor cells (29, and unpublished observations). While wild-type HIV-1 replication was incompatible with rhabdomyosarcoma proliferation, deletion of Vpr from the HIV-1 genome allowed the maintenance of a population of cells which proliferated in the presence of active HIV-1 replication (29), leading to the proposition that through dysregulation of cellular proliferation and differentiation, Vpr may contribute to HIV pathogenesis. Another prediction resulting from that study was that via its effects on cells, Vpr regulates cellular permissiveness to HIV replication.

Vpr protein is found in virions of HIV-1, HIV-2, and SIV (6, 67, 68). The incorporation of Vpr protein into the viral particle provides both a mechanism for delivery of Vpr protein into cells at the time of infection and also a means of export of Vpr from infected cells. Here we report that recombinant Vpr protein, when added to cell culture medium at a low concen-

tration, is a powerful activator of HIV replication in new infection of transformed and primary cells as well as in latently infected cell lines. In latently infected cell lines, Vpr protein increases HIV expression at a translational or pretranslational stage. Further, extracellular Vpr protein establishes a pool of efficient targets of HIV replication, as cells exposed to Vpr protein produce increased virus when infected several days after exposure to Vpr.

MATERIALS AND METHODS

Plasmids and cloning: construction of *vpr* baculovirus vector. A fragment from the pBABE*puro-vpr* vector previously described (29, 30), containing the *vpr* open reading frame of HIV-1 NL43 with the consensus eukaryotic ribosome binding site, was excised using *Bam*H-I and then ligated into *Bam*H-I-cut baculovirus vector pVL1393 (Invitrogen). pVL1393 is derived from the *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) and uses the polyhedrin promoter (P_{polh}) for expression of cloned inserts. pVL1393-*vpr* DNA and linearized Ac*M*NPV DNA (BaculoGold, Pharmingen) were transfected into SF9 (*Spodoptera frugiperda*) cells (Invitrogen). To provide control virus not coding for the Vpr protein, pVL1393 vector lacking an insert was transfected along with BaculoGold DNA in a manner identical to that described above and all subsequent expression procedures were carried out as for Vpr expression. Viral stocks used for further infection of insect cells were prepared after plaque purification of recombinant baculoviruses.

Insect cell culture. SF9 were grown as adherent cells in Costar T-75 flasks in Grace's modified insect medium (Gibco) supplemented with yeastolate (Gibco)–lactalbumin hydrolysate (Gibco)–10% fetal calf serum (FCS)–penicillin-streptomycin, and fungizone (Sigma) at 25°C. High Five cells (*Trichoplusia ni*) (Invitrogen) were grown in SF-900 medium (Gibco) supplemented as above except without FCS.

Mammalian cell culture. TE671 embryonal rhabdomyosarcoma cells (ATCC HTB 139) (39, 61) were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle medium plus 10% FCS–penicillin-streptomycin–sodium pyruvate–25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.4) at 37°C in a humidified 5% CO_2 atmosphere. Uninfected H9 (52), SupT-1 (58), and U937 cells and the latently infected lines J1.1 (derived from Jurkat [48]), ACH-2 (derived from an A3.01 clone of CEM [14]), OM.10.1 (derived from HL60 [4]), U1 (derived from U937 [15]), and LL58 (derived from X50.7, an Epstein-Barr virus-transformed B-lymphoblastoid cell line [9]) were obtained from the National Institutes of Health AIDS Reagent Program (NIH ARP). KG-1 was obtained from Georgio Trinchieri. HL60 was purchased from the ATCC. THP-1 was kindly provided by the Medical Research Council AIDS Directed Programme Reagent Project. Peripheral blood mononuclear cells (PBMC) were isolated from a healthy HIV-negative donor by Ficoll-Hypaque centrifugation. All primary and transformed cells were cultured at 37°C in a humidified 5% CO_2 atmosphere in RPMI 1640 supplemented with 10% heat-inactivated FCS (or 10% autologous human serum for PBMC)–penicillin-streptomycin–2-mercaptoethanol–25 mM HEPES (pH 7.4).

Protein expression. Recombinant baculovirus, either containing (Vpr) or lacking (control) the *vpr* gene, from transfected SF9 cells was used to infect High Five cells at a multiplicity of infection of 5 to 10. High Five cells were grown prior to infection in T-75 flasks and then transferred to a 250-ml spinner flask 1 day before infection and grown as described above. Only healthy (>95% viability) log-phase growth cultures at a cell density of 2×10^6 cells/ml were infected for protein production. At peak protein production (24 h postinfection), supernatants were collected and centrifuged to remove particulates, and protease inhibitors were added (aprotinin, leupeptin, pepstatin A, each at 2 μg/ml; phenylmethylsulfonyl fluoride, 1 mM; EDTA, 1 mM). Supernatants were then dialyzed against three changes of 100 volumes of 10 mM Tris (pH 8.5) with 10 μM 2-mercaptoethanol (Sigma). Dialyzed supernatants were filtered through a 0.45-μm-pore-size membrane and then supplemented with HEPES (pH 7.4) to 25 mM. Vpr and control supernatants prepared in this manner were stored on ice until use. Vpr and control preparations were mycoplasma and endotoxin negative. No infectious baculovirus remained after these procedures, as determined by an infection plaque assay.

Preparation of LR1 rabbit anti-Vpr serum. Recombinant Vpr was purified to about 80% purity by immunoaffinity chromatography using 808 rabbit anti-Vpr serum (obtained from B. Cullen through the NIH ARP [19]), followed by DEAE-Sepharose chromatography. This product was used to immunized a rabbit three times. A fourth and fifth immunizations were performed using three Vpr peptides from the amino and carboxyl termini and the central portion of Vpr (HIV-1 NL43 Vpr amino acids 9 to 20, GPQREPYNYWTL; 41 to 55, SLGQH IYETYGDTWA; and 81 to 96, HFRIGCRSHRIGITRQRRARNGASRS) (purchased from American BioTechnologies) coupled to keyhole limpet hemocyanin. LR1 rabbit anti-Vpr serum recognized recombinant Vpr, Vpr from HIV-1-infected cells, and Vpr in the serum of HIV-positive individuals in enzyme-linked immunosorbent assay (ELISA) and Western immunoblot and did not react with any other cellular or viral proteins. All sera were heat inactivated for use in tissue culture.

Purification of Vpr by immunoaffinity chromatography. LR1 immunoglobulin G was coupled to protein G-agarose beads using DMP (22). Dialyzed baculovirus culture supernatant (Vpr containing or control) was passed through the LR1-protein G immunoaffinity column, and then the column was washed extensively with phosphate-buffered saline (PBS) plus 0.5% Triton X-100. Three bed volumes of preelution buffer consisting of 10 mM sodium phosphate (pH 8.0)–0.5% Triton X-100 was passed through, followed by elution buffer consisting of 100 mM triethanolamine (pH 11.5) plus 0.5% Triton X-100. The eluted fractions were collected in 1/20 volume of 1 M sodium phosphate (pH 6.8). For use in tissue culture, Vpr-containing fractions were passed through a detergent removal column (Pierce), dialyzed extensively against PBS, filter sterilized, and stored at 4°C until use. Purified Vpr was used where indicated; alternatively, dialyzed Vpr-containing culture medium was applied at a final concentration of 400 pg/ml. Control column eluate was used as a control for purified Vpr, and control insect culture medium was used as a control when Vpr-containing culture medium was applied. Neither of the negative control preparations (purified protein or baculovirus supernatants) displayed any activity in more than 25 independent experiments. Quantification of purified Vpr was performed by Coomassie staining of sodium dodecyl sulfate (SDS)-polyacrylamide gels, using known amounts of protein molecular weight standards for comparison of band intensity. The Vpr content of baculovirus supernatants was measured by titration in ELISA, using serial dilutions of purified Vpr as a standard. Purified Vpr and Vpr-containing baculovirus supernatants displayed identical activity in HIV replication studies per picogram of Vpr.

Virus stocks and infection of cells with HIV-1. Virus stocks were obtained from the NIH ARP, thawed once, aliquoted, and then stored at −80°C until use. An HIV-1 *vpr* deletion mutant, NL43Δvpr, has been previously described (29). For Vpr mutation studies, wild-type NL43 and Vpr mutant NL43Δvpr were produced following transfection of SupT-1 cells by electroporation. Virus stocks were normalized for virus content for infection. Infection of cells was performed by incubating virus with cells under standard tissue culture conditions for 12 h and then washing cells with growth medium to remove unbound virus. Typically, 100 50% tissue culture infective doses ($TCID_{50}$) were used to infect 50×10^3 to 100×10^3 cells. No effect on either infection efficiency or Vpr response was observed by using from 10 $TCID_{50}$ to 6×10^4 $TCID_{50}$. Polybrene had no effect in this assay. No p24 antigen was detectable in supernatants following washing of cells. Culture medium was replaced as required by cell growth, equally to all wells.

p24 and Vpr antigen capture ELISA. $p24^{gag}$ antigen was measured by capture ELISA, using a monoclonal antibody in solid phase (V7.8, obtained from Evan Hersh through the NIH ARP) and polyclonal sheep anti-p24 (obtained from Michael Phelan through the NIH ARP) followed by a peroxidase-coupled anti-sheep antibody (Boehringer Mannheim) for detection of bound antigen. Commercial recombinant p24 (ABT) was titrated as a standard curve, and the response was linear on log plots over the range of 0.2 pg/ml to 10 ng/ml. Sensitivity was 2 to 10 pg/ml in tissue culture supernatants owing to a dilution of 1:10 used in the assay. Standard deviation in ELISA duplicates was typically <1% to 10%.

Fluorescence staining, flow cytometry, and photomicrography. Prior to being stained, cells were fixed with 80% ethanol–10% acetic acid at −20°C for 10 min and then washed with FACS buffer (Hanks balanced salt solution, 5% horse serum, 0.1% sodium azide, 25 mM HEPES [pH 7.4]). The fixed cells were then incubated with a cocktail of anti-p24 mouse monoclonal antibodies, washed extensively with FACS buffer, incubated with either anti-mouse rhodamine-conjugated antibody (for micrography; Boehringer Mannheim) or anti-mouse fluorescein isothiocyanate (for flow cytometry; Boehringer Mannheim), and then washed extensively with FACS buffer. Cells were fixed with 1% paraformaldehyde immediately after being stained. Immunofluorescence photomicrography was performed using a microscope, color-charged coupled device camera, and video and video printing devices kindly loaned to us by Kristin Kelley and Optical Apparatus, Inc. The original magnification was ×200.

RESULTS

Expression of recombinant HIV-1 Vpr. Native HIV Vpr was produced in insect cells after infection with recombinant baculovirus containing the *vpr* open reading frame cloned from the infectious HIV-1 molecular clone NL43. Surprisingly, the vast majority of Vpr was found in the culture supernatant of recombinant baculovirus-infected insect cells rather than in association with the cells. The recombinant Vpr was identical to Vpr from HIV-infected cells in migration on SDS-polyacrylamide gel electrophoresis (Fig. 1A). Recombinant Vpr had a reactivity identical to that of viral Vpr in Western immunoblot and ELISA with anti-Vpr serum and HIV-positive patient serum. Anti-Vpr serum (LR1) was produced in rabbits after

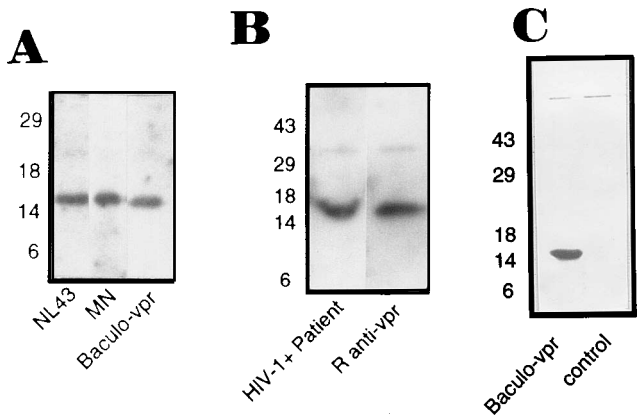


FIG. 1. Recombinant Vpr and reactivity of anti-Vpr antisera. (A) Vpr-containing recombinant baculovirus supernatants and lysates of HIV-1-infected cells (NL43 and MN strains) were run on SDS–15% polyacrylamide gels, transferred for Western blot, and probed with anti-Vpr peptide serum 808. (B) Recombinant Vpr was run and transferred as described for panel A and then probed with either HIV-positive patient serum or with anti-Vpr serum from a recombinant Vpr-immunized rabbit. (C) Purification of recombinant Vpr. Recombinant Vpr was purified by immunoaffinity chromatography, and then 5 μg was loaded on an SDS–15% polyacrylamide gel which was then silver stained. Left lane, eluate from Vpr-containing baculovirus supernatant; right lane, eluate from control baculovirus supernatant.

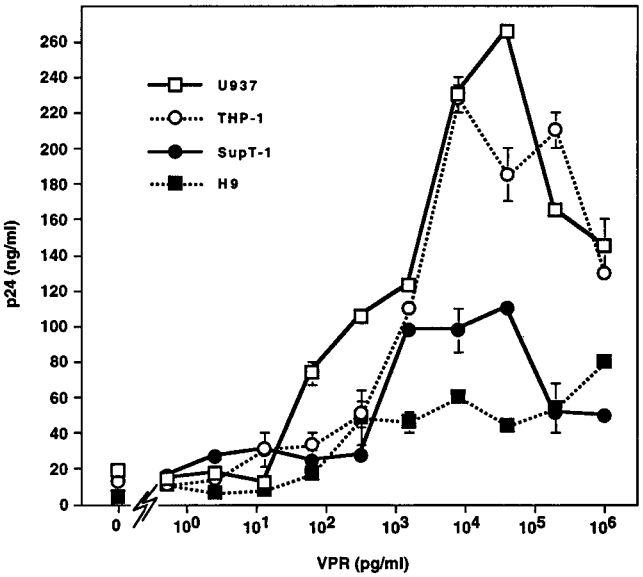


FIG. 2. Dose response of purified recombinant Vpr in HIV-1 infection. The indicated cell lines were infected with HIV-1 in the presence of various amounts of purified recombinant Vpr protein. At 12 h after infection and Vpr exposure, the cells were washed to remove residual virus and Vpr; they were then cultured under standard conditions. $p24^{gag}$ protein was assessed on day 6 by capture ELISA as described in Materials and Methods. p24 values and standard deviations were computed from duplicate cultures.

immunization with partially purified protein and Vpr peptides (Fig. 1B).

Recombinant Vpr protein displays biological activity. The presence of most of the Vpr protein in the insect cell supernatant was intriguing and prompted us to investigate the possibility that this protein displayed biological activity as an extracellular molecule. Continuous exposure of TE671 rhabdomyosarcoma cells to Vpr induced growth inhibition and cell differentiation as had transfection with the *vpr* gene (29, 30) (not shown).

We chose to first investigate whether soluble Vpr protein could affect the replication of wild-type HIV, for this might have relevance to infection with wild-type viruses in vivo. Recombinant Vpr protein was purified by using immunoaffinity chromatography (Fig. 1C). Purified recombinant Vpr displayed migration and antibody reactivity identical to that of the crude recombinant protein preparation or native viral Vpr. We exposed four cell lines, two promonocytic lines (THP-1 and U937) and two T-lymphoid lines (H9 and SupT-1), to concentrations of 0.5 pg/ml to 1 μg/ml of purified Vpr at the time of infection with HIV-1 NL43, washed out both the virus and Vpr after 12 h, and then assayed the presence of new virus in the culture medium. Virus production from each cell line was assessed on day 6 by p24 ELISA for each amount of purified Vpr to which the cells were exposed (Fig. 2). The two promonocytic cell lines showed an increase in virus production beginning at less than 100 pg of Vpr per ml for the U937 cell line and around 300 pg/ml for the THP-1 promonocytic cell line. Peak virus production was found using 600 to 2,000 pg of Vpr per ml for each cell line, with an enhancement of 10- to 15-fold in virus production by day 6. Similar data were obtained with two other cell lines, HL60, a promonocytic/erythrocytic line, and the promonocytic line KG-1. The T-lymphocytic cell lines also responded to Vpr treatment with an increase in virus production. A decrease in Vpr response was found with doses greater than 4×10^4 to 10×10^4 pg/ml with the exception of the H9 T-lymphoid cell line, which was the least responsive to Vpr. H9 cells continued to show increased virus replication at doses up through 1 μg of Vpr per ml.

Virus production could be detected in the culture medium of the Vpr-treated cells typically 1 day earlier than from the untreated cells and continued to be 10 to 20 times higher for about 7 days after infection (Fig. 3). To further test whether the increase in virus replication was solely an effect of the Vpr protein, U937 and SupT-1 cells were exposed to 320 pg of Vpr per ml at the time of infection in the presence of anti-Vpr antibodies, control antibodies, or medium alone. The Vpr effect was completely inhibited by anti-Vpr antibodies but was not affected by control immune serum (Fig. 3). LR1 anti-Vpr serum also inhibited Vpr activity. After about 1 week following infection and exposure to Vpr, virus production from Vpr-treated and untreated cells was found to be the same, indicating that Vpr functioned to increase the rate of virus production in culture, consistent with previous findings using *vpr* mutant viruses that Vpr increases replication kinetics (24, 44, 56, 57).

Extracellular Vpr protein complements a *vpr* mutant HIV-1. We investigated whether extracellular Vpr protein would restore replication to a *vpr* deletion mutant virus. Wild-type NL43 or the *vpr* mutant NL43Δvpr (29) was used to infect SupT-1 or U937 cells in the presence or absence of Vpr. NL43 and NL43Δvpr replicated equally in the T-lymphocytic Sup-T1 cell line, and the extracellular Vpr-treated cells displayed equal increases in virus expression for each virus (Fig. 4). Similar results were obtained with the H9 T-lymphocytic cell line (not shown). On the other hand, NL43Δvpr replicated poorly in the U937 monocytic line. Extracellular Vpr restored the replication of the Vpr mutant in this cell line; in fact the replication of the Vpr mutant plus Vpr was faster in the early days than was that of the wild-type virus without extracellular Vpr but not as fast as that of wild-type virus plus Vpr. Similar results were obtained with the THP-1 monocytic cell line. This result suggests that both endogenously produced (virally encoded) Vpr and exogenous Vpr were active in monocytic infection but that only extracellular Vpr appeared to contribute to HIV replication in the T-cell lines.

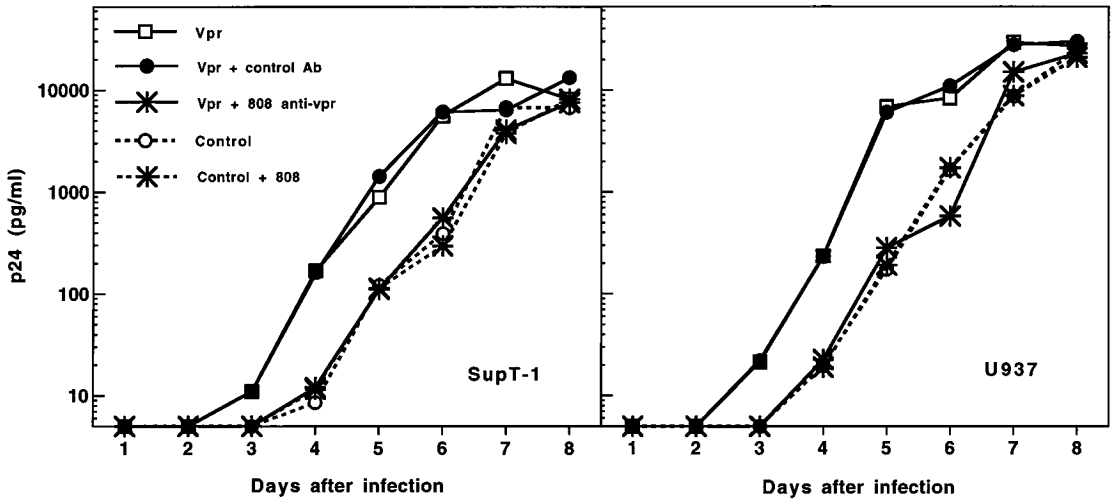


FIG. 3. Time course of HIV infection and antibody inhibition of Vpr activity. SupT-1 T-lymphoblastic cells or U937 monocytoid cells were exposed to 320 pg of purified Vpr per ml at the time of infection with HIV-1 NL43. In addition, some wells received rabbit anti-Vpr serum or an irrelevant rabbit immune serum. Virus production was assessed by p24 ELISA on culture supernatants collected on the indicated days after infection and exposure to agents. p24 values represent mean values from duplicate cultures.

The Vpr effect is aimed at the cellular targets of infection. Vpr may directly assist virus replication through an interaction with other viral constituents, or it may regulate cellular events involved in virus replication, or both. The ability of the *vpr* gene to regulate the proliferation and differentiation of various cell types (29) suggested that Vpr may affect cellular permissiveness to productive HIV infection. In order to test this theory, cells were exposed to either Vpr or control protein for 12 h and the cells were then washed and rested in normal culture medium for 5 days prior to infection (Fig. 5). In each case, the Vpr-treated cells displayed an increase in HIV production identical to treatment of the cells at the time of infection. Cells cultured for up to 1 week after Vpr treatment (the longest time tested) retained increased permissiveness to HIV replication. Interestingly, the Vpr mutant virus replicated significantly better in the Vpr-treated monocytic cells than in the control cells, paralleling the results obtained in monocytic cells treated with Vpr at the time of infection.

Vpr is active in primary hematopoietic cells. We next examined the activity of extracellular Vpr on natural targets of HIV infection. PBMC from a HIV-negative donor were infected with HIV-1 in the presence of the cell activators phytohemagglutinin (PHA) and interleukin-2 (IL-2) or Vpr or a combination of each for 12 h (Fig. 6); then all agents and infecting virus were washed from the cultures. This transient treatment with PHA and IL-2 failed to increase HIV-1 replication; however, HIV-1 replication was significantly greater in the Vpr-treated PBMC. The combination of Vpr and PHA-IL-2 yielded greater virus production than either alone, indicating that the suboptimal exposure to PHA-IL-2 did affect the cells in a manner that could be enhanced by Vpr.

Vpr activates virus from latently infected cells. Cultures of

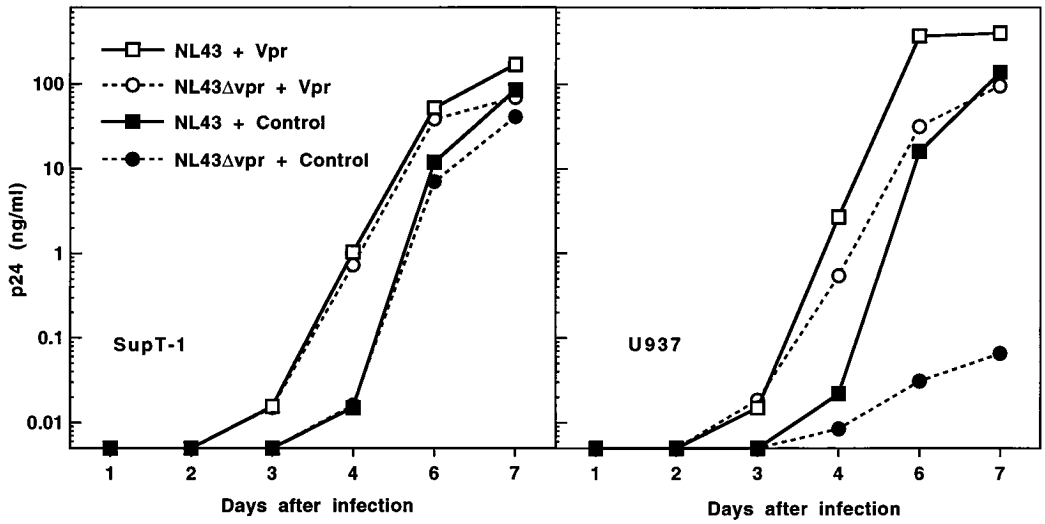


FIG. 4. Extracellular Vpr complementation of a Vpr deletion mutant HIV-1. Cells were infected with either wild-type HIV-1 NL43 or the Vpr mutant HIV-1 NL43Δvpr at the time of exposure to purified Vpr (320 pg/ml). Other procedures were performed as described in the legend to Fig. 3.

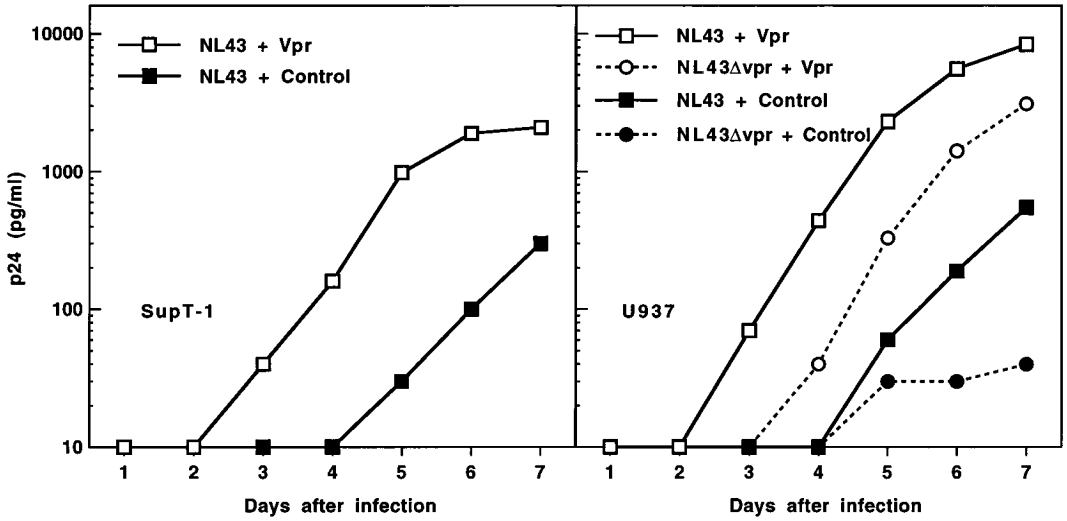


FIG. 5. Vpr induction of a long-lasting permissiveness to HIV replication. Cells were exposed to 320 pg of purified Vpr per ml for 12 h, washed, and then cultured in normal growth medium for 5 days prior to infection. Other procedures were performed as described in the legend to Fig. 3.

cells infected with HIV often spontaneously decline in virus production and enter what is variously termed nonproductive, silent, or latent infection. This tendency of HIV to establish chronic low-level expression may be an important factor in viral persistence in vivo. We next investigated whether Vpr could rescue HIV expression in such cells. A culture of HIV-1-infected THP-1 monocytic cells, from which measurable virus production had declined to undetectable levels 2 weeks after infection, was exposed to soluble Vpr or to the phorbol ester phorbol myristate acetate (PMA) for the first time on day 20 after infection. PMA stimulates HIV transcription through activation of protein kinase C. Virus export resumed after a single exposure to Vpr or PMA, and significant levels of virus production continued for at least 3 weeks following treatment (Fig. 7). The levels of virus replication achieved in the Vpr-stimulated cultures were severalfold greater than those of the PMA-stimulated cultures. Similar reactivation of HIV-1 expression was obtained by using nonproductively infected cultures of U937 and HL60 cells, and in each case Vpr induction was three- to fivefold greater than PMA induction (not shown). In addition, in some experiments, virus export could be measured in the culture supernatant as soon as 14 h after Vpr exposure.

Several well-characterized, latently infected clonal cell lines have been developed as in vitro models of cellular latency (4, 8, 14, 15, 48). These cell lines come from multiple investigators, and with one exception (LL58), each has been shown to resume or increase virus expression after exposure to phorbol ester or tumor necrosis factor alpha. Virus expression has also been induced in the U1 line by IL-6 (49), gamma interferon (49, 59), granulocyte-macrophage colony-stimulating factor

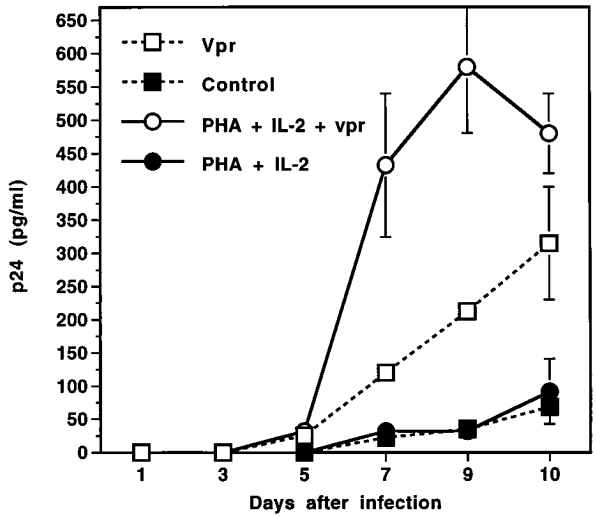


FIG. 6. Vpr increases HIV-1 replication in primary PBMC. PBMC from a healthy HIV-negative donor were prepared by Ficoll-Hypaque centrifugation and then cultured in the presence of the indicated agents for 12 h at the time of infection. PHA, 5 μg/ml; IL-2, 50 U/ml; Vpr, 400 pg/ml. Other procedures were performed as described in the legend to Fig. 3.

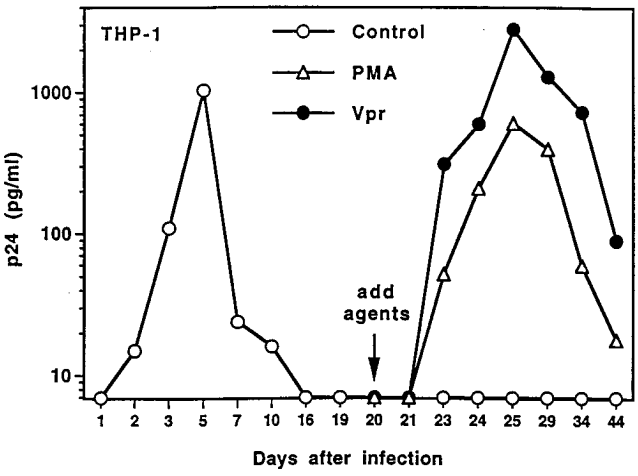


FIG. 7. Reactivation of HIV-1 expression from a spontaneously quiescent culture. THP-1 monocytoid cells were infected with HIV-1 NL43 in normal growth medium and then maintained in normal growth medium (open circles up to day 19). Measurable virus production ceased after day 10. On day 20, Vpr (400 pg/ml) or PMA (50 ng/ml) was added to the cells, which were then cultured in normal growth medium. Vpr, closed circles; PMA, open triangles. Control supernatants had no effect on HIV expression (open circles after day 20).

(15), or heat (59). These cell lines are derived from both T-cell and monocyte lineages, including two parental lines used in the above infection studies (OM.10.1 is derived from HL60 and U1 is derived from U937). The LL58 line is derived from an Epstein-Barr virus-transformed B-lymphocytic line.

Each cell line was exposed to Vpr protein and then examined for virus expression. Intracellular staining for p24 antigen revealed that Vpr reactivated virus protein expression in the cells (Fig. 8). Similar results were obtained with each of the latently infected cell lines. Flow cytometric analysis of p24 staining demonstrates that in all five latently infected cell lines, virus production was reactivated or increased in >90% of the cells (Fig. 9). The two T-cell lines (ACH-2 and J1.1) and one myeloid line (U1) demonstrated constitutive intracellular p24 expression, though extracellular virus was not detected in the culture medium of unstimulated cells. In the LL58 line about 10% of untreated cells were antigen positive, but virus expression was found in >90% of Vpr-treated cells. Virus export was induced in all five cell lines by Vpr (Fig. 10) and was inhibitable with each of the anti-Vpr antibodies (not shown). PMA activated HIV replication in these cell lines to levels similar to that induced by Vpr (not shown).

DISCUSSION

The results presented here demonstrate that Vpr protein is active as an extracellular molecule acting to increase HIV replication. Purified Vpr protein functioned at very low concentrations, was active on the two main cell types which are targets of HIV infection (T-lymphocytic cells and monocytic cells), but was most active in new infection in monocytic cells, consistent with published Vpr mutation studies. Extracellular Vpr was inhibited by anti-Vpr antibodies. In addition to restoring monocyte replication competence to a Vpr mutant virus, extracellular Vpr boosted the replication kinetics of wild-type HIV-1. Extracellular Vpr rendered cells more permissive to HIV replication, and this highly permissive state lasted several days. In addition to assisting HIV replication in transformed cell lines, extracellular Vpr was active in primary PBMC. Extracellular Vpr reactivated HIV expression in latently infected cell lines which, when uninduced, express no or low levels of HIV RNA and protein.

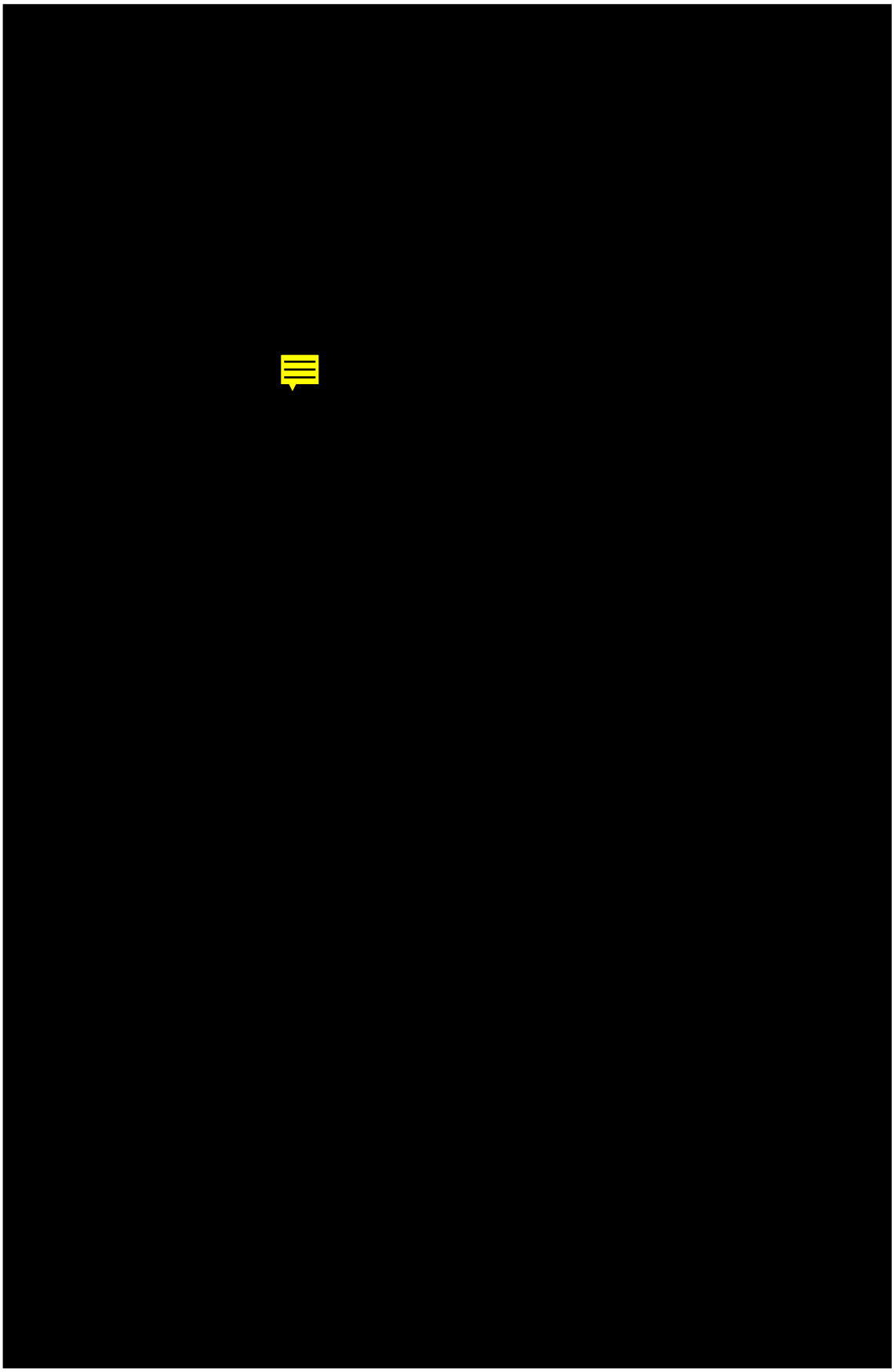
There are numerous precedents for viral proteins with activity as extracellular molecules. Several of these viral proteins mimic or interact with cellular growth factors, oncogenes, or cytokines, each of which influences cell replication or differentiation (reviewed in reference 10). For example, Epstein-Barr virus protein BCRF1 has properties very similar to those of IL-10 and is believed to interact with the immune system and thus promote virus replication and survival (26). Two human retroviral transactivating proteins, HIV-1 Tat and human T-cell leukemia virus type I Tax, are active in soluble, cell-free form (16, 21, 36, 65). Tat has been shown to enter hematopoietic cells and transactivate the HIV long terminal repeat (65). In addition, extracellular Tat can promote cellular growth, indicating a cellular pathway for activity which may be separate from its HIV transactiving function. However, extracellular Tat and Tax are not generally activators of viral replication.

Our previous findings that Vpr, in the context of the virus or when expressed by itself, inhibits the growth of cells and activates the committed cellular differentiation program of muscle tumor cells led to the proposition that Vpr might assist HIV replication via activation of cellular programs which determine cellular permissiveness to virus replication (29). The effects of Vpr on cellular proliferation may be a secondary effect of the regulation of cellular permissiveness, or HIV replication may be more efficient in cells whose growth is specifically arrested. We favor the former interpretation because cellular proliferation generally increases HIV replication. In this report we find a long-lasting alteration in cellular permissiveness to HIV replication. This highly permissive phenotype was induced in both the T-lymphocytic and the monocytic lines. This is a further demonstration that Vpr can act in the absence of other HIV elements, such as Gag proteins, which may be important for additional reported Vpr functions. Vpr may elucidate the expression of cellular factors which assist HIV expression, such as transcription factors, or suppress the expression of cellular factors which would inhibit HIV replication (29). We found that extracellular Vpr increased the replication of HIV in resting PBMC in the absence of other cell stimulators; thus, since Vpr protein did not induce cell proliferation in these cells (data not shown) and the *vpr* gene is cytostatic in several cell types (29, 30), Vpr may alter the cellular phenotype to facilitate replication of HIV in nonproliferating cells.

The similarities between the activities of Vpr protein on latently infected cells and some features of latency in another pathogenic viral system, that of herpes simplex virus (HSV), are striking. HSVs contain within their structures (viral tegument) several virally encoded proteins which appear to be involved in the control of latency (reviewed in reference 18). It has been proposed that the tegument protein brought into cells during infection influences the decision between lytic and latent HSV infection. Tegument proteins ICP0 and Vmw110 are capable of reactivating virus expression in latently infected cells through transcriptional activation of early HSV genes, including themselves, in a probable positive feedback loop (23, 72). In support of this analogy, HIV-2 Vpx protein is structurally and evolutionarily similar to Vpr (42, 63, 64), and virion-associated Vpx has been shown to increase the efficiency of viral replication immediately after infection (27). Thus, HIV may have adopted mechanisms for regulating productive versus latent infection similar to those of classical latent viruses such as HSV.

Recent studies have shown that Vpr may assist early events in HIV infection (71). We have shown here that extracellular Vpr increases virus protein production in latently infected cells, which involves late postintegration events in HIV replication (transcription and translation). The latently infected cell lines ACH-2 and U1 have been found to express very low levels of only fully spliced message prior to induction with cytokines (51). Activation of virus expression requires induction of viral transcription and export of unspliced and singly spliced viral messages coding for the viral structural proteins. Cohen et al. have shown that transfection of Vpr into cells containing reporter constructs can increase transcription from HIV and other promoters by about threefold (7). Our previous findings that Vpr inhibits cell growth and can activate cellular differentiation are also indications that Vpr may affect transcriptional events either directly or indirectly. The Vpr activity

FIG. 8. Activation of virus expression in latently infected cells. (A) Promonocytic line OM.10.1. (B) T-lymphocytic line ACH-2. Cells were exposed to 400 pg of Vpr per ml for 2 days and on day 4 following exposure were stained for intracellular $p24^{gag}$ protein. The left panels were recorded under white light; the right panels show $p24^{gag}$ expression by immunofluorescence. The top two panels of each group of four represent control-treated cells; the bottom two panels of each group show Vpr-treated cells. Untreated cells displayed results identical to those of control-treated cells.



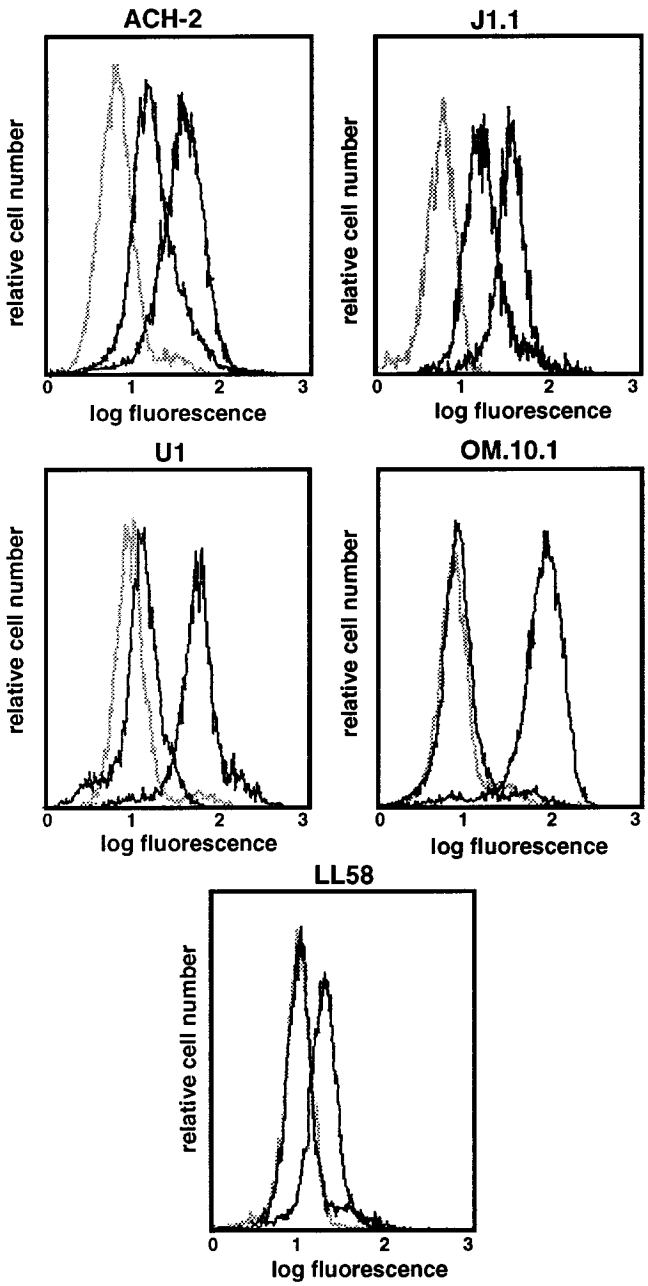


FIG. 9. Flow cytometric analysis of Vpr-treated latent cell lines. Anti-p24 immunofluorescence was performed as described in the legend to Fig. 8 on the latently infected cell lines U1, OM.10.1, ACH-2, J1.1, and LL58. In each histogram, the lightly stippled curve represents background staining with secondary antibody alone. The leftmost black curve in each case represents control-treated cells, and the far right black curve represents Vpr-treated cells. Untreated cells presented staining identical to that of control-treated cells.

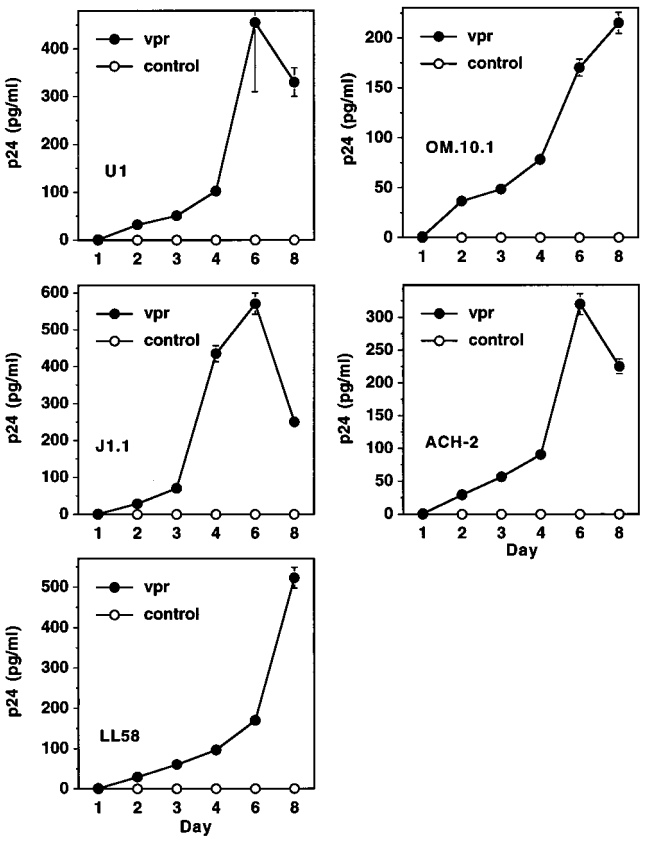


FIG. 10. Rescue of virus expression from latently infected clonal T lymphocytes, B lymphocytes, and monocyte lines. Five latently infected cell lines were exposed to 400 pg of Vpr per ml on day zero. p24 content of the culture medium was assessed as described in the legend to Fig. 3. Standard deviations were computed from duplicate cultures.

reported here might explain transcellular activation of the HIV long terminal repeat observed after coculture of HIV-infected cells with target cells containing reporter constructs (38). The experiments reported here do not indicate whether Vpr assists early or late HIV replication events in newly infected cells. However, since Vpr assists late events in cells which are already (latently) infected, we prefer the notion that extracellular Vpr activates late events in newly infected cells as well. This concept has the advantage of simplicity plus the added attraction of unifying the pathway for Vpr enhancement of HIV expression in latently and productively infected cells through a regulation of cellular permissiveness to HIV replication.

The long-lasting increase in cell permissiveness reported here indicates that Vpr may create a "pool" of highly permissive potential targets of infection. Since Vpr is specifically incorporated into the viral particle in amounts similar to those of the Gag proteins (6), Vpr is exported from infected cells at a high level. Virus disintegration or immune lysis of virions could release Vpr into bodily fluids where autocrine or paracrine regulation of HIV replication would ensue. We have recently found biologically active Vpr in the serum and cerebral spinal fluid of HIV-infected individuals in levels that correlate with the degree of p24 antigenemia observed and disease state (30a). The high level of virus replication that occurs after initial infection, and also at the last stage of disease, may be accelerated by a positive feedback mechanism driven by free extracellular Vpr. Extracellular Vpr could also provide a means to reactivate virus expression in latently infected cells in vivo. The majority of infected peripheral blood lymphocytes do not express measurable amounts of viral antigens and must be stimulated in vitro with mitogens or other activators in order to express virus (2, 3, 17, 32, 53). The percentage of infected cells in the lymphoid tissues is much greater than that in the peripheral circulation (45, 46), and recently it has been reported that large amounts of latently infected cells persist in these tissues throughout the course of HIV infection (11, 12, 46).

The ability of Vpr to activate both new virus expression and virus export in all latently infected cell lines tested suggests that Vpr could activate virus expression in areas, such as lymphoid tissues, in which high local concentrations of viral antigens are found. Since Vpr also regulates the proliferation and development of diverse cell types, extracellular Vpr might contribute to tissue-specific pathologies associated with HIV infection, including wasting, neurological disease, and T-cell depletion.

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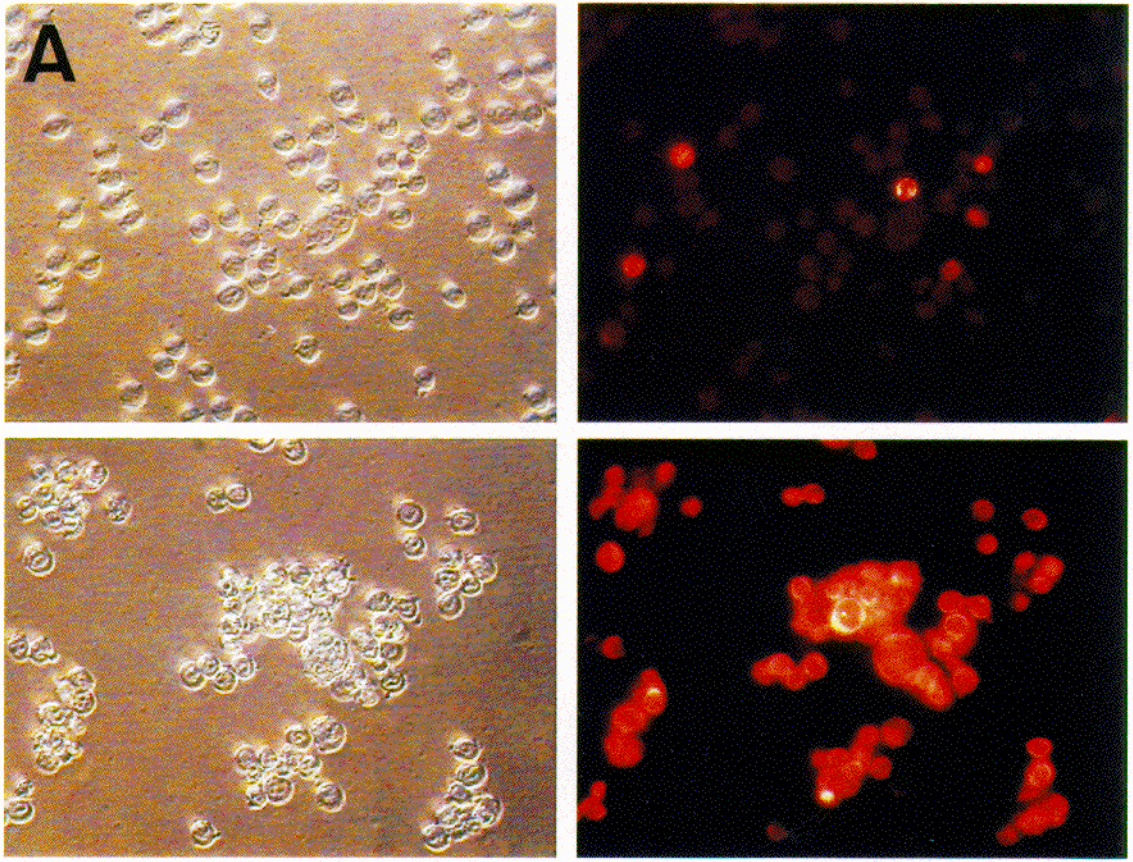
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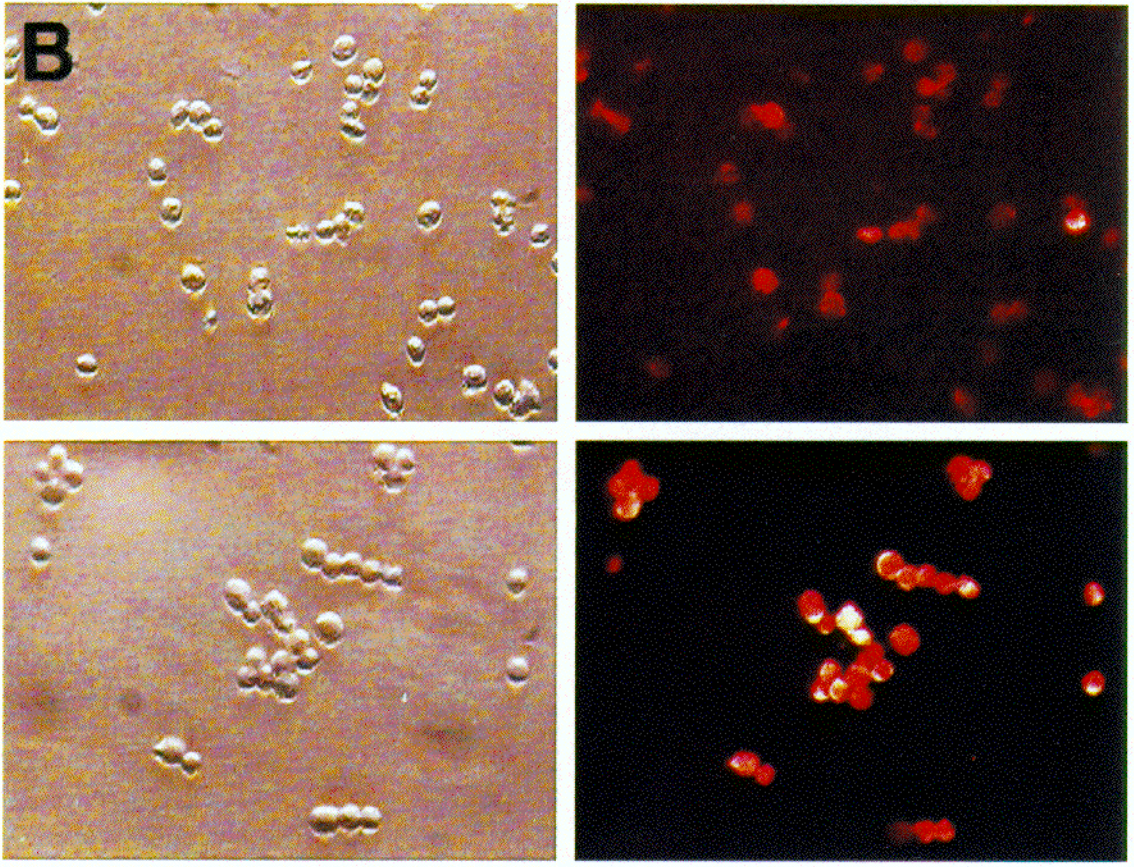
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