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Interferon-α But Not AZT Suppresses HIV Expression in Chronically Infected Cell Lines

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Promonocytic (U1) and T lymphocytic (ACH-2) cell lines chronically infected with human immunodeficiency virus type 1 (HIV-1) constitutively express low levels of virus, but expression can be induced by phorbol esters and cytokines. Whereas ACH-2 cells produce infectious virions, U1 cells produce defective, noninfectious particles. Although 3′-azido-3′-deoxythimidine (AZT) prevented acute HIV infection of susceptible cells, it did not prevent the induction of HIV expression in the infected cell lines. In contrast, interferon alpha (IFN-α) inhibited the release of reverse transcriptase and viral antigens into the culture supernatant after phorbol ester stimulation of both cell lines. Further, IFN-α suppressed the production or release (or both) of whole HIV virions, but had no effect on the amount of cell-associated viral proteins. Also, after phorbol ester stimulation of ACH-2 cells, IFN-α reduced the number of infectious viral particles secreted into the culture supernatant, but had no effect on the infectivity of cell-associated virus. These findings lend support to the combined use of antiviral agents that have action at both the early (AZT) and the late (IFN-α) stages of HIV replication.

ACQUIRED IMMUNODEFICIENCY SYNdrome (AIDS) is characterized immunologically by depletion and functional impairment of the CD4-positive lymphocyte subset (*1*). Other cells of the immune system, such as B lymphocytes (*2*) and mononuclear phagocytes (*3*), are also functionally impaired, and can be targets of human immunodeficiency virus (HIV) infection (*4*). The onset of clinical symptoms often occurs years after initial infection with HIV (*5*), suggesting that a state of latency or permanent expression of virus exists for variable periods in the majority of patients. In support of this hypothesis, in vitro observations indicate that HIV can persist in both lymphocytic (*6, 7*) and monocytic (*8*) cells for long periods in a low replicative or latent form until activation signals trigger viral expression. Among the inductive signals associated with HIV replication in vitro are antigens (*9*), mitogens (*7, 10*), phorbol esters such as phorbol myristate acetate (PMA) (*11, 12*), and cytokines (*13, 14*), cellular transcription factors such as Sp1 (*15*) and NF-κB (*16*), and transfected genes of other viruses (*17*). Furthermore, monocytes and tissue macrophages are relatively resistant to the cytopathic effect of HIV (*3, 8*), suggesting that they might serve as circulating and tissue reservoirs of the virus. Therefore, the development of in vitro models reflecting the different stages of HIV infection (that is, acute compared to latent or chronic infection) in different cell types could be relevant to the design of broader and more effective antiviral strategies. In this regard, 3′-azido-3′-deoxythymidine (AZT) (*18*) and recombinant interferon alpha (IFN-α) (*19*) have shown antiretroviral activity and clinical efficacy in HIV-infected patients. In addition, IFN-α has shown efficacy in the treatment of AIDS-associated Kaposi's sarcoma (*19, 20*). Whereas AZT inhibits reverse transcription of viral RNA, an early phase of the viral cycle, and therefore prevents the integration of HIV provirus into the host genome (*21*), little is known about the mechanism of action of IFN-α on HIV in susceptible cells. Studies of murine retroviruses indicate that IFNs inhibit early events, such as transcription or translation of the viral genome during acute infection (*22*). However, when chronically infected cells were studied, IFNs predominantly affected the late stages of virion release from the plasma membrane (*22*).

Previously we developed two cell clones permanently infected with HIV-1. One clone (U1) was obtained by limiting dilution of cells surviving the acute infection of the promonocytic cell line U937. A second clone (ACH-2) was similarly derived from cells surviving the acute infection of the T lymphocytic cell line A3.01. The U1 and the ACH-2 cell clones have, respectively, two and one integrated copies of proviral DNA as determined by restriction enzyme analysis (*23*). In addition, both clones express low or undetectable constitutive levels of HIV.

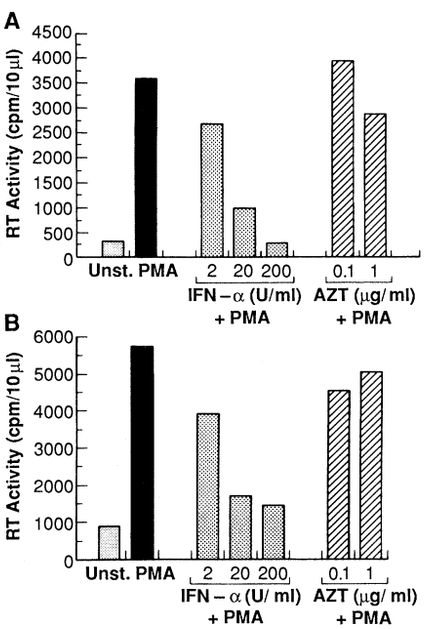

Fig. 1. Effect of IFN-α and AZT on HIV expression in chronically infected cells. Promonocytic U1 cells (**A**) and T lymphocytic ACH-2 T cells (**B**) were resuspended at 2.5×10^5 per milliliter in RPMI 1640 supplemented with 10% fetal calf serum. Cells were then stimulated with PMA ($10^{-7}M$) in the presence or absence of recombinant IFN-α (2 to 200 U/ml) or AZT (0.1 to 1 μg/ml) for 48 hours at 37°C in 7% CO_2. Unstimulated cells (unst.) were incubated under the same conditions. Culture supernatants were then harvested and tested for the presence of HIV RT activity (*33*). Briefly, 10 μl of supernatants were added in duplicate to 50 μl of a mixture containing polyadenylate, oligo(dT) (Pharmacia), $MgCl_2$, and [α^{32}P]-labeled deoxythymidinetriphosphate (dTTP) (Amersham) and incubated for 2 hours at 37°C. A sample (10 μl) of the mixture was then spotted on DE81 ion exchange chromatography paper (Whatman), air-dried, washed five times in 2× standard saline citrate buffer, and twice more in 95% ethanol. The paper was then dried, cut, and the radioactivity present was determined in a Beckman LS 7000 scintillation counter. Variability of replicate cultures was always less than 10%. The patterns represent more than ten independent experiments.

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Expression of HIV is strongly inducible however in both cell clones by various agents, such as PMA and cytokines (*12–14*). Therefore, we compared the ability of AZT and recombinant human IFN-α to affect PMA-induced HIV expression in U1 and ACH-2 cells.

We found that IFN-α inhibited the PMA-induced expression of HIV in U1 and ACH-2 cells, reducing the level of reverse transcriptase (RT) activity in the supernatant of both cell lines (80 to 90% for U1 and 50 to 75% for ACH-2). The effective concentration range of IFN-α was between 20 and 200 U/ml (Fig. 1). Antiretroviral activity of IFN-α was maximal when IFN-α and PMA were added simultaneously; when cells were incubated with IFN-α for 24 hours before PMA stimulation no significant improvement in antiretroviral efficacy was seen. Kinetic studies showed that the antiretroviral effects of IFN-α were still present when IFN-α was added to U1 and ACH-2 cultures 30 hours after PMA stimulation (with a reduction of supernatant RT activity of approximately 50 and 30%, respectively) (*24*). We did not see a significant effect of AZT (0.1 to 1 μg/ml) on PMA-stimulated HIV expression in U1 or ACH-2 cells (Fig. 1). Higher concentrations (5 and 10 μg/ml) of AZT reduced U1 supernatant RT levels, but this decrease could be accounted for by drug-induced cytotoxicity (>50% cell death, as evaluated by Trypan blue dye exclusion). The ACH-2 cells were more resistant to AZT-induced cytotoxicity, but there was still no detectable effect of AZT on HIV expression at these higher concentrations of drug. However, AZT inhibited HIV infection of A3.01 cells in a dose-dependent manner; at a concentration of 10 μg/ml, the drug blocked HIV expression without inducing cell toxicity (>80% viable cells after 18 days of culture), whereas at 0.1 or 1 μg/ml a significant delay (10 to 12 days) in the peak of viral infection as measured by RT was observed (*24*).

The amount of cell-associated RT after PMA stimulation was not affected by IFN-α treatment in U1 and ACH-2 cells (Fig. 2). Similar results to those found for RT were obtained by measuring the amount of p24 antigen released in the supernatant or associated with IFN-α–treated U1 and ACH-2 cells (*25*). These data suggest that the inhibitory activity of IFN-α was mainly affecting post-transcriptional stages of the viral cycle. To further test this interpretation we evaluated the expression of HIV proteins in IFN-α–treated U1 and ACH-2 cells by indirect immunofluorescence and protein blot analysis. Immunofluorescence revealed that IFN-α treatment did not reduce the percentage of cells expressing HIV proteins after PMA stimulation (>90%); the intensity of fluorescence evaluated on a per cell basis was also not affected. Protein blot analysis of U1 and ACH-2 cell lysates confirmed that IFN-α treatment did not suppress the quantitative synthesis or the qualitative profile of the expressed HIV proteins (Fig. 3).

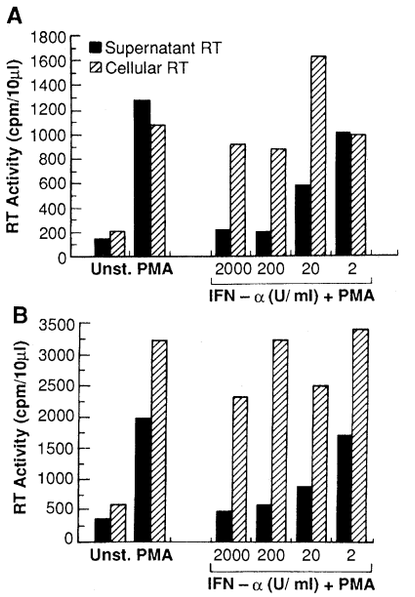

Fig. 2. Supernatant and cell-associated RT activity of (**A**) U1 and (**B**) ACH-2 cells. Cells were incubated in the presence or absence of PMA ($10^{-7}M$) and IFN-α (2 to 2000 U/ml) for 48 hours as described (Fig. 1). The RT activity of culture supernatants and cell lysates was then evaluated. Cells were disrupted in the original volume of fresh culture medium by exposure to five cycles of freezing and thawing. Each bar represents mean RT levels from triplicate cultures (less than 15% variability per culture) from a representative experiment. Five independent experiments were performed. The RT activity in disrupted IFN-α treated cells was always >75% of that in untreated control cells.

Ultrastructural studies of untreated U1 and ACH-2 cells and cells treated with PMA in the presence or absence of IFN-α revealed characteristic HIV morphology (Fig. 4). The addition of IFN-α to PMA-treated cells did not obviously affect the percentage of cells assembling viral particles as indicated by virus budding or the presence of mature particles closely associated with the cell membrane (up to 90% of cell sections were virus-positive, in some experiments). Treatment of unstimulated cells with IFN-α did not significantly alter the percentage of virus-positive cells. As we previously observed (*12*), U1 cells treated with PMA also assembled virus within cytoplasmic vacuoles consistent with Golgi-derived multivesicular bodies, a feature not shared by ACH-2 cells (*26*). Although we had already demonstrated by RT determinations (Figs. 1 and 2) and protein blot analysis (Fig. 3) that IFN-α did not significantly affect the amount of cell-associated viral protein, the total number of viral particles associated with the average cell section appeared to increase by two- to threefold in IFN-α treated cells (Fig. 4). In U1 cells the increase was detectable both at the plasma membrane and within intracytoplasmic vacuoles. These findings agree with studies on murine cells permanently infected with murine leukemia virus (MuLV), which demonstrated that a non-immune IFN was primarily inhibiting the release of preformed virions from the plasma membrane (*22*). The inhibition of release of MuLV particles after IFN treatment was described as a "post-budding effect" to indicate an apparent increase of virions present in clusters on the external side of the plasma membrane. One possible mechanism of IFN-α action on chronically HIV-infected cells is that IFN-α affects the expression of one or more viral components that are required for the efficient assembly and release of progeny virions. One such component could be the *vpu* gene product (*27*). On the other hand, the observation that IFNs exert similar effects on cells permanently infected with MuLV suggests that regulatory genes typical of human and simian retroviruses, such as *vpu*, are not involved in the mechanism of action of IFN-α. Another possibility is that the accumulation of virions on the surface of cells treated with IFN-α is related to a decreased fluidity of the plasma membrane, as suggested for the murine system (*28*). Cells expressing more HIV particles on the cell surface may represent better targets for cytotoxic T lymphocytes (*29*). Moreover, IFN-α can activate mononuclear phagocytes and natural killer cells in vitro to exert cytotoxic function not restricted by the major histocompatability complex (*30*). The accumulation of virions in association with the plasma membrane of the infected cell after IFN-α treatment could also lead to the release of viral particles when the drug is withdrawn. This phenomenon has been observed in vitro in an acute infection system (*31*), and we observed such a rebound phenomenon in patients treated with IFN-α after the drug was discontinued (*19*).

We found that the infectious titer of HIV in the culture supernatant of PMA-stimulated ACH-2 cells was reduced by approximately 60% when the cells were incubated with IFN-α at 200 U/ml. However, the infectivity of cell-associated virus was not affected by IFN-α treatment (*24*).

Ho *et al.* observed that IFN-α inhibited the acute HIV infection of peripheral blood lymphocytes (*32*); at the same time IFN-α treatment reduced the percentage of cells infected by HIV, as determined by specific immunofluorescence. This last observation

is in contrast to our findings on U1 and ACH-2 infected cells, which did not show any reduction in the percentage of immunofluorescent positive cells after PMA stimulation in the presence of IFN-α. These different responses of acutely and permanently infected cells to IFN-α suggest that IFN-α can probably affect additional steps in the virus life cycle earlier than the release of mature virions from the plasma membrane.

Thus, the demonstration that IFN-α is effective in directly preventing the release of HIV from permanently infected cells provides a rationale to design experimental protocols of combined antiretroviral therapy in which a drug such as AZT, which blocks de novo infection of cells with HIV, is administered together with IFN-α, which blocks virion release from chronically or latently infected cells.

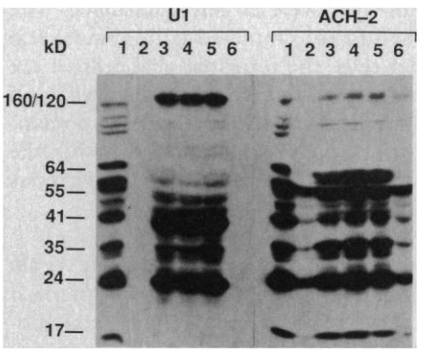

Fig. 3. Immunoblot of U1 and ACH-2 cells after IFN-α treatment. Cells were incubated in the presence or absence of PMA and IFN-α for 48 hours, as described (Fig. 1). Lysates of 2×10^6 cells in 50 ml of buffer were prepared (*34*), and samples (20 μl) were subjected to electrophoresis through 3 to 27% gradient polyacrylamide gels (Integration Separation Sciences, Hyde Park, MA) for 6 hours. The separated proteins were transferred overnight onto nitrocellulose filters. After saturation with a 5% milk solution, filters were incubated for 2 hours with serum from an AIDS patient (1:1000 dilution). Filters were washed and then incubated with ^{125}I-labeled protein A (200,000 dpm/ml), washed, air-dried, and exposed overnight to x-ray film, as described (*34*). (Lane 1) The pattern of HIV protein expression from infected A3.01 cells (*34*) is shown for comparison. (Lane 2) Unstimulated cells. (Lane 3) Cells stimulated with PMA ($10^{-7}M$). (Lanes 4 and 5) Stimulated cells treated with IFN-α at 200 and 2000 U/ml, respectively. (Lane 6) Unstimulated cells treated with IFN-α at 200 U/ml.

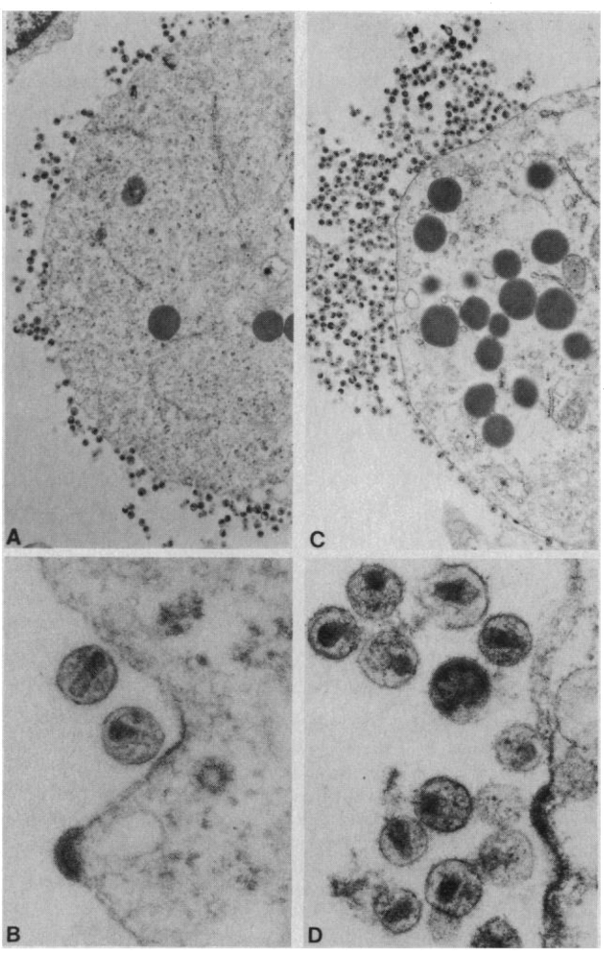

Fig. 4. Low (**A** and **C**) and high (**B** and **D**) magnification transmission electron micrographs showing relative proportions of plasma membrane-associated HIV for ACH-2 cells treated with $10^{-7}M$ PMA for 48 hours in the absence (A and B) and presence (C and D) of IFN-α (200 U/ml) (Fig. 1). Morphologically, the virus appeared indistinguishable at all stages of assembly and maturation (B and D). Typically, virus production appeared to preferentially take place on the plasma membrane on the Golgi side of the cell. Varying amounts of cytoplasmic lipid were seen in cells in all preparations. (A and C, ×12,000; B and D, ×100,000). Cultured cells were isolated by centrifugation, washed in buffer, fixed in glutaraldehyde and then in OsO_4, dehydrated in graded ethanol and propylene oxide, and embedded in plastic as described (*12*).

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