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Alpha Interferon-Induced Antiretroviral Activities: Restriction of Viral Nucleic Acid Synthesis and Progeny Virion Production in Human Immunodeficiency Virus Type 1-Infected Monocytes

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Alpha interferon (IFN-α) restricts multiple steps of the human immunodeficiency virus type 1 (HIV-1) life cycle. A well-described effect of IFN-α is in the modulation of viral nucleic acid synthesis. We demonstrate that IFN-α influences HIV-1 DNA synthesis principally by reducing the production of late products of reverse transcription. The magnitude of IFN-α-induced downregulation of HIV-1 DNA and/or progeny virion production was dependent on the IFN-α concentration, the duration of cytokine administration, the multiplicity of infection, the viral strain, and the cycles of viral infection. Interestingly, reductions in viral DNAs could not fully account for the observed IFN-α-induced abrogation of progeny virion production. These data, by our investigation of both single-cycle and spreading viral infections, support a predominant but not exclusive effect of IFN-α on viral DNA synthesis.

Interferons (IFN) are important regulatory molecules for host antiretroviral responses and viral pathogenesis (7, 10, 30, 31). Indeed, IFN is a first-line defense mechanism against many animal viruses, including human immunodeficiency virus type 1 (HIV-1) (28, 30, 31). IFN activity in serum during acute HIV seroconversion correlates with diminished levels of virus and rising $CD4^+$ T-cell numbers (28). These antiviral actions of IFN are mediated by sets of IFN-inducible genes that affect viral restriction but surprisingly remain poorly defined for HIV-1 (11, 31, 33). Paradoxically, levels of alpha IFN (IFN-α) in serum or surrogate markers for IFN activity (neopterin, β2-microglobulin, 2′,5′-oligoadenylate, and/or tubuloreticular inclusion bodies) correlate with HIV-1 plasma viremia and progressive immunosuppression during the later stages of viral infection (10). Thus, IFN plays a role both in viral restriction and in pathogenesis during HIV-1 infection.

IFN is efficacious for treatment of patients with early-stage HIV infection and those with Kaposi's sarcoma (6, 17, 23, 24). Marked antiviral activities of IFN follow addition of IFN-α, -β, and -γ to HIV-1-infected T cells and monocytes (1, 10, 12, 13, 17, 19, 26, 30–34). How the HIV-1 life cycle is affected by IFN treatment remains incompletely defined, and experimental results vary among investigators. A review of these studies suggests that IFN's actions are dependent on the in vitro laboratory system, the viral strain, the target cells, the multiplicity of viral infection (MOI), the assay systems for virus detection, and the type, concentration, and dosing intervals of the IFNs (10, 30). To control for these variables and to better define the mechanism of action of IFN against HIV-1, we used a well-characterized monocyte cell system and varied the viral strain, the MOI, and the concentrations of IFN to quantitate viral nucleic acid synthesis and its transport to the host cell nucleus during IFN treatments. We demonstrate that IFN's action is mediated, in part, against viral reverse transcription. Differences between viral isolates were quantitative and primarily related to the virus' ability to replicate in monocytes rather than on differences in IFN's ability to restrict one isolate more than another. The dosage of IFN in culture systems also determined the level of viral restriction. Interestingly, quantitative reductions in viral nucleic acids could not completely explain the abrogation of progeny virion production observed in IFN-treated cells, indicating that IFN-α also influences viral replication at stages beyond viral nucleic acid synthesis (1, 10, 13). These results support the argument for potential use of IFN in therapies designed to restrict specific stages of the HIV life cycle.

The ability of IFN-α to affect HIV infection in monocytes was analyzed with two monocytotropic strains ($HIV\text{-}1_{ADA}$ and $HIV\text{-}1_{DJV}$) (16, 20) in primary human monocytes (Fig. 1). Both $HIV\text{-}1_{ADA}$ and $HIV\text{-}1_{DJV}$ elicited a spreading infection in monocytes. However, after the addition of 50 to 5,000 IU of IFN-α per ml 18 h prior to HIV inoculation (with continuous exposure of the cells to IFN-α through 15 days), an abrogation of progeny virion production (supernatant reverse transcriptase [RT] activity) was observed. Levels of RT activity were reduced for $HIV\text{-}1_{DJV}$ compared with $HIV\text{-}1_{ADA}$ levels at identical MOIs and without IFN-α treatment. Nevertheless, the responses to IFN-α for both viral strains were similar. These effects could not be explained by induction of IFN-α or -β by HIV-1, since no IFN-α or -β activity was detected in culture fluids of monocytes infected with each of the two viral strains during the course of infection (reference 13 and data not shown). IFN activity in culture fluids was assayed by inhibition of cytopathic effects induced by vesicular stomatitis virus in MDBK cells. Differentiation between IFN-α and -β was performed by using their respective neutralizing monoclonal antibodies placed into the MDBK cells together

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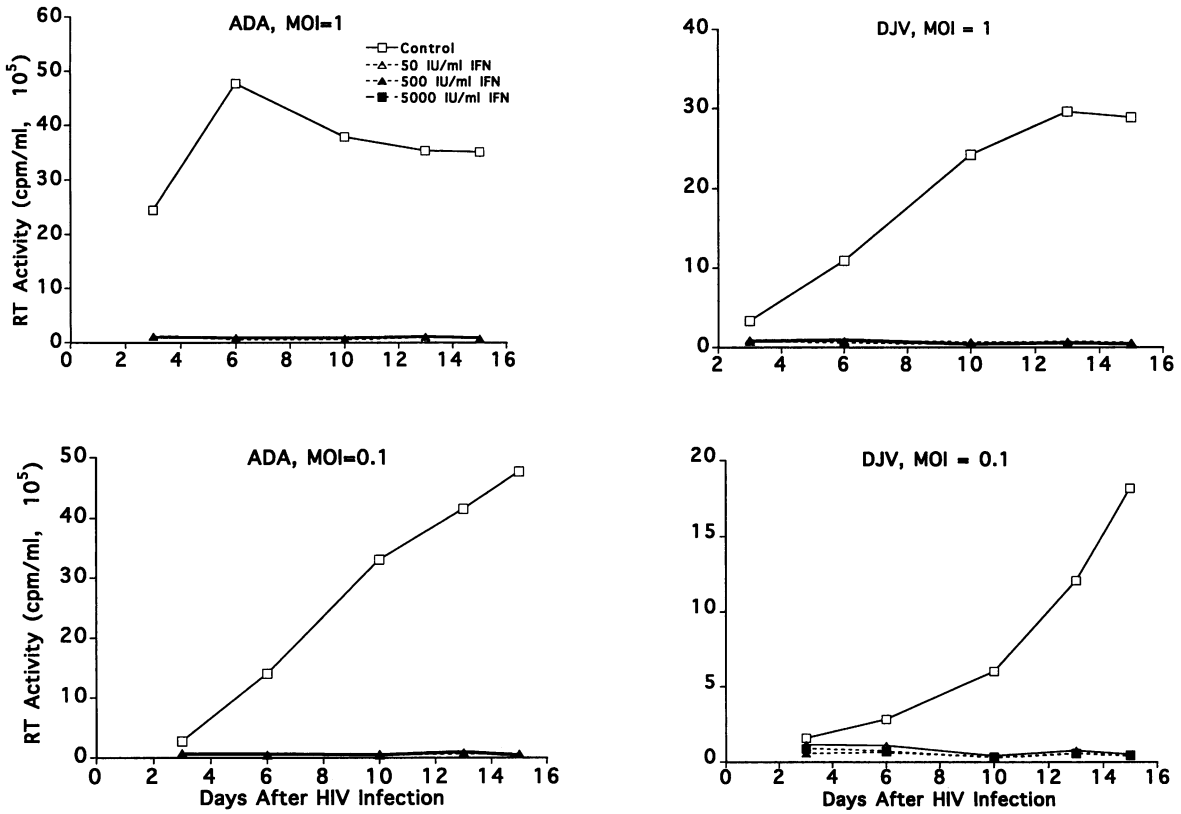

FIG. 1. Effects of continuous IFN-α treatment on replication of HIV in monocytes. Peripheral blood mononuclear cells obtained by leukopheresis were purified to >98% monocytes by centrifugal elutriation (16) and cultured for 7 days as adherent monolayers in medium with human sera and macrophage colony-stimulating factor, a generous gift from Genetics Institute, Cambridge, Mass. Macrophage colony-stimulating factor was removed from the medium, and cells were exposed for 18 h to recombinant IFN-α_{2b} (Schering Plough, Kenilworth, N.J.) from 0 to 5,000 IU/ml. Eighteen hours after IFN inoculation, the cells were exposed to either HIV-1_{ADA} or HIV-1_{DJV} at the MOI of 1 or 0.1. MOI measurements were obtained by inoculating 10-fold dilutions of viral stock onto 7-day-cultured monocytes. All titrations were done in quadruplicate. After 21 days postinoculation, viral replication was scored as positive if two of the following three criteria were met in 50% of the wells: (i) the presence of virus-induced cytopathic effects (multinucleated giant cells and cell lysis), (ii) p24 antigen levels in culture fluids of ≥5 ng/ml, and (iii) RT activity in culture fluids of $\geq 10^5$ cpm/ml. For the data shown, IFN was maintained throughout the experimental time intervals. All cultures were refed with fresh medium every 2 to 3 days. For RT activity, replicate samples of culture fluids were added to a reaction mixture of 0.05% Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.), poly(A) (10 μg/ml), oligo(dT) (0.25 U/ml; Pharmacia, Piscataway, N.J.), 5 mM dithiothreitol (Pharmacia), 150 mM KCl, 15 mM $MgCl_2$, and ^{3}H-dTTP (2 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) in Tris-HCl buffer (pH 7.9) for 24 h at 37°C. Radiolabeled nucleotides were precipitated with cold 10% trichloroacetic acid and washed with 5% trichloroacetic acid and 95% ethanol in an automatic cell harvester (Skatron Inc., Sterling, Va.) on glass filter discs. Radioactivity was estimated by liquid scintillation spectroscopy (22). HTLV-IIIB served as positive control for the RT activity.

with the experimental culture fluids or IFN control samples (1, 13).

We next determined whether the kinetics of progeny virion production could be influenced by altering IFN-α administration (Fig. 2). In these experiments, cells received a single dose of IFN-α, 50 to 5,000 IU/ml, 18 h prior to viral inoculation. Monocytes were maintained in culture medium without exogenous IFN-α during a 15-day observation period. These experiments demonstrated that the duration of IFN-α treatment played an important role in its ability to restrict viral replication. Indeed, a single dose of IFN-α (50 to 5,000 IU/ml) 18 h prior to virus infections showed a reduction but not abrogation of RT activity. In this case, virus production was delayed by 3 to 5 days for both infections. Eventually, both viruses were able to overcome the antiretroviral effects of IFN-α. These data indicate that the antiretroviral effects of IFN are contingent upon the duration of IFN-α treatment. The levels of viral replication of either of the monocytotropic viral strains in monocytes did not affect the antiretroviral effects or breakthrough of virion production following single-dose IFN-α therapy.

We next examined whether the abrogation of virion production by IFN-α was mediated by commensurate reductions in rates of viral nucleic acid synthesis and its nuclear import. Synthesis of early, intermediate, and late products of RT were identified by PCR with primers to long terminal repeat (LTR) U3/R, *pol*, LTR R/*gag*, and LTR U5 regions of the viral genome (3–5). The RT inhibitor zidovudine was also used to assist in distinguishing de novo from preexisting virion-associated DNA (25, 35) (data not shown). Definitive evidence for the presence of full-length plus- and minus-strand viral DNAs was confirmed by analyzing the synthesis of two LTR circle forms of episomal viral DNA. Although these episomal forms of viral DNA do not represent provirus precursors, they are formed only after synthesis of full-length viral DNA and its transport to the host cell nucleus (2–5). Accumulation of early, intermediate, and late products of reverse transcription including circle forms of viral DNA were thus examined in HIV-1-

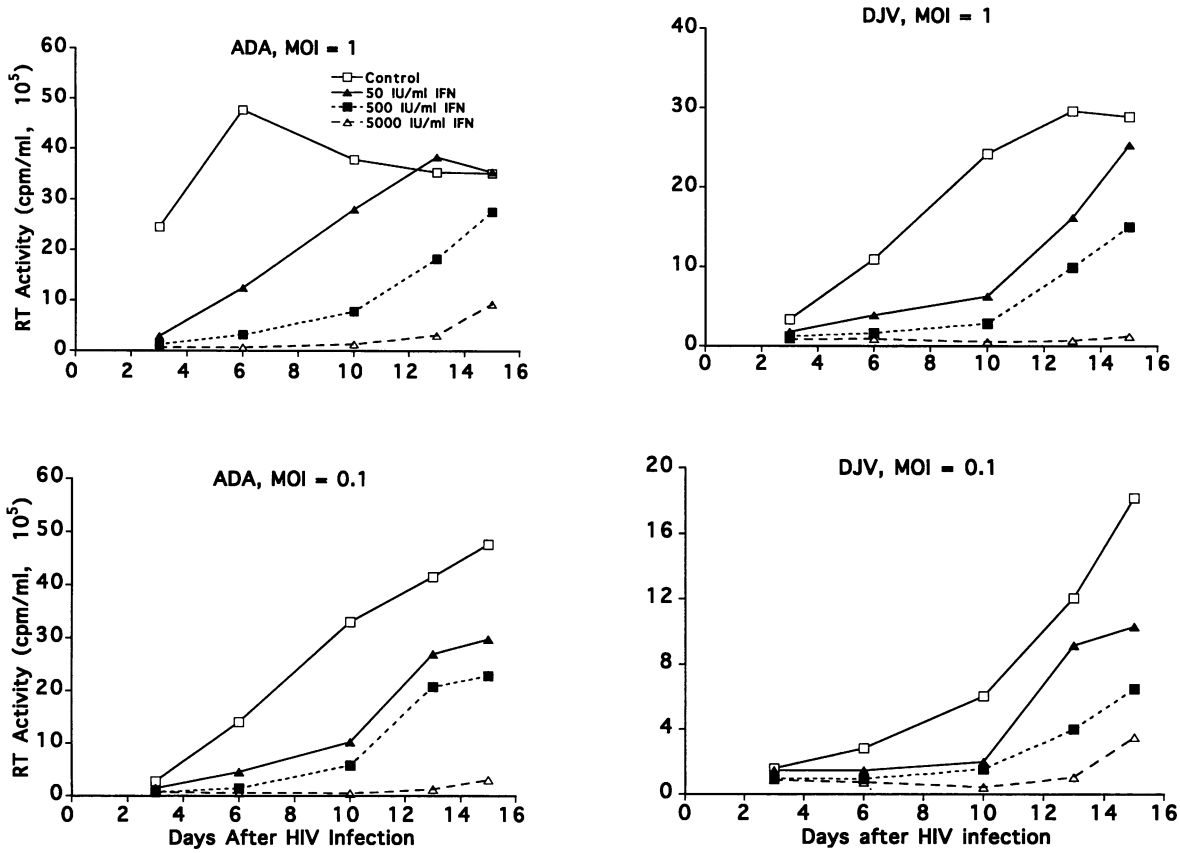

FIG. 2. Effects of single-dose IFN pretreatment on the replication of HIV in monocytes. Peripheral blood mononuclear cells obtained by leukopheresis were purified to >98% monocytes by centrifugal elutriation and cultured for 7 days as adherent monolayers in medium with human sera and macrophage colony-stimulating factor. After 7 days, the macrophage colony-stimulating factor was depleted and the cells were exposed for 18 h in a single inoculation with 0 to 5,000 IU of recombinant IFN-α. IFN was given as a single dose and was not maintained in the culture fluids throughout the observation period. Eighteen hours after IFN treatment, the cells were inoculated with either HIV-1_{ADA} or HIV-1_{DJV} at an MOI of 1 or 0.1 infectious virus per cell. All cultures were refed with fresh medium every 2 to 3 days. RT activity in culture supernatants was determined as described in the legend for Fig. 1.

infected cells during IFN-α treatment. De novo synthesis levels of early products of reverse transcription were similar for both HIV-1_{DJV}- and HIV-1_{ADA}-infected monocytes and quantitatively reduced after IFN-α (50 to 5,000 IU/ml) treatment. Modest changes (Fig. 3A; Table 1) were shown after treatment with ≤5,000 IU of IFN-α, indicating that no marked inhibition of early stages in virus replication (binding, fusion, or strong stop DNA synthesis) occurred in the presence of IFN (Fig. 3). Synthesis of intermediate and late reverse transcription intermediates was affected by IFN-α treatments in both HIV-1_{ADA}- and HIV-1_{DJV}-infected monocytes (Fig. 3). More pronounced reduction of intermediate and late products of reverse transcription than of early viral cDNA products was evident for both HIV-1_{DJV} and HIV-1_{ADA}. The reduction in abundance of intermediate and late products of reverse transcription in the presence of IFN was more pronounced at a lower MOI (Fig. 3B; Table 2). In this instance, viral nucleic acid synthesis and its transport to the monocyte nucleus were almost abrogated by cell treatments of ≥500 IU of IFN-α_1.

The contribution of virion-associated DNA (35, 36) was excluded by comparing levels of viral DNA synthesis in monocytes in the presence and absence of zidovudine. The episomal forms of viral DNA containing two LTR circles were detected within 24 h postinfection in both HIV-1_{ADA}- and HIV-1_{DJV}-infected cells (Fig. 3A); in the lower-MOI assay (Fig. 3B), these episomal forms were detected after 48 and 72 h in HIV-1_{ADA}- and HIV-1_{DJV}-infected cells, respectively. This result suggests that under the culture conditions and MOI used in this study viral DNA synthesis was complete within 72 h. Little virion-associated DNA was observed in cultures at early times postinfection, and as a consequence, little if any viral DNA accumulation was observed in monocyte cultures treated with zidovudine (data not shown). Early, intermediate, and late products of reverse transcription were evident by 8 h postinfection with HIV-1_{ADA}, and accumulation of late products of RT were delayed in HIV-1_{DJV}-infected cells. We do not attach much significance to the observed quantitative differences in reverse transcription between the two isolates and conclude that viral DNA synthesis proceeds rapidly to completion in monocytes infected with both viral strains. The inhibition of synthesis of the intermediate and late products of reverse transcription was dependent on the IFN-α dose. This inhibition was more marked with the HIV-1_{DJV} strain, suggesting altering responses of specific viral strains to IFN-α. Quantitatively, the extent of viral cDNA synthesis, as measured by copy numbers, in the presence of IFN-α, is summarized in Tables 1 and 2. Here, HIV-1 copy numbers within virus-infected monocytes treated with IFN-α for 48 h (Fig. 3), were

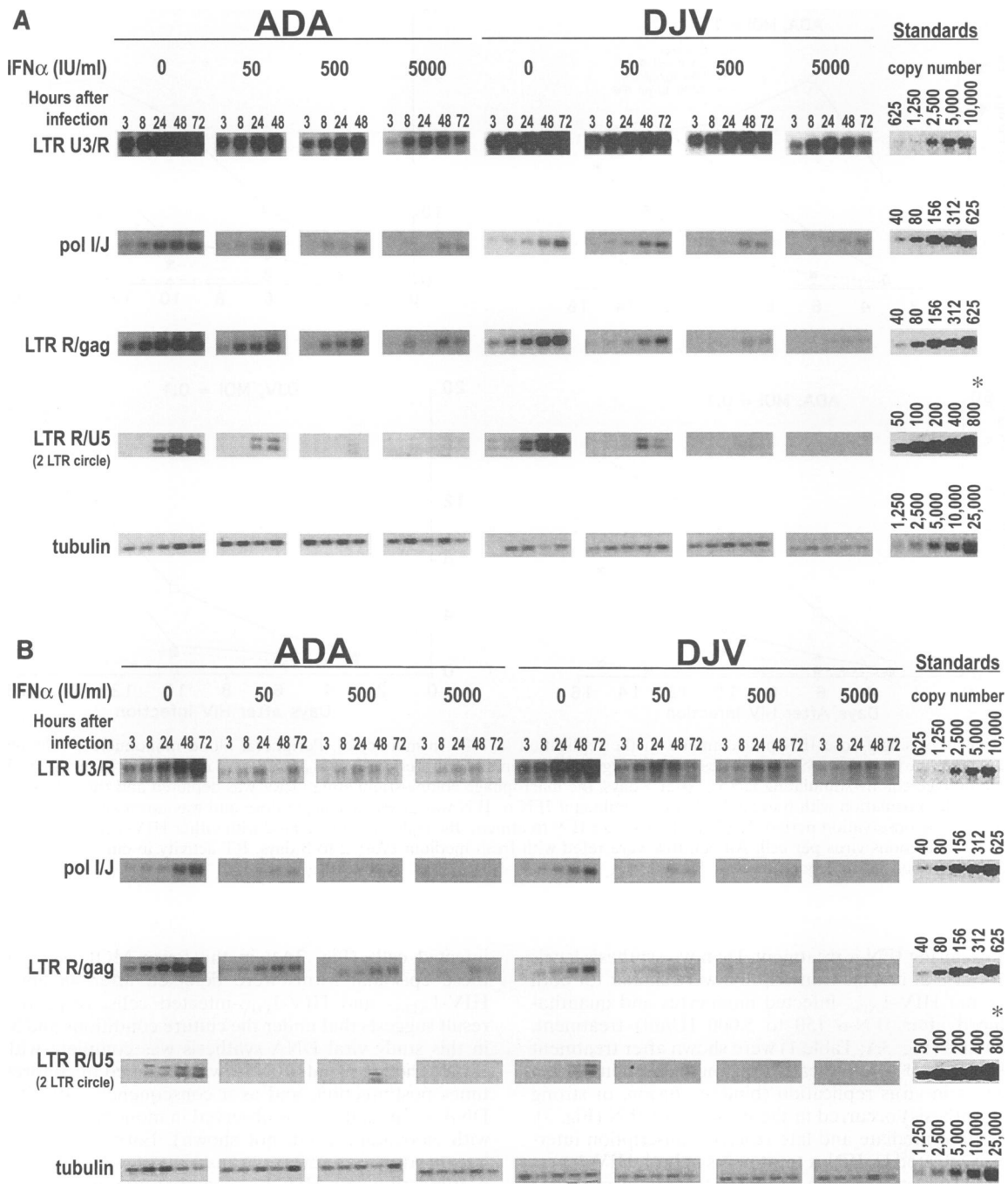

FIG. 3. Synthesis of viral DNA in HIV-1-infected monocytes following IFN treatment. Monocytes were infected with HIV-1 at an MOI of 1 (A) or 0.1 (B). At the indicated times postinfection, monocytes were removed by scraping from Costar six-well plates (initially seeded with 3×10^6 monocytes per plate) for isolation of total cellular DNA and analysis by PCR. From 0 to 5,000 IU of IFN-α was added to monocyte cultures 18 h prior to infection and was maintained in the culture medium throughout the period of experimental analyses. DNA from cells obtained from one well of the six-well Costar plate was subjected to 30 cycles of PCR with primers to LTR U3 and R (coordinates 9194 to 9214 and 9591 to 9610 of HIV HXB2 (3–5); *polI* and *polJ* (coordinates 2131 to 2149 and 2592 to 2610, respectively). LTR R and *gag* (coordinates 9194 to 9214 and 794 to 815, respectively) for amplification of early, intermediate, and late reverse transcription products, respectively. Amplification of episomal forms of viral DNA containing two LTRs was performed by using LTR R and U5 (coordinates 9591 to 9610 and 9650 to 9679, respectively) primers. For the LTR primers, only 3′ LTR coordinates are given. PCR amplification products were visualized after Southern blot transfer and hybridization with ^{32}P-labeled oligonucleotide probes (20). Hybridized blots were visualized on a molecular Phosphorimager SF (Molecular Dynamics). Standards for LTR U3/R, *polI/J*, and LTR R/*gag* products were generated by PCR on doubling dilutions of DNA from 8E5 cells which contain one defective viral genome per cell (9). Standards for the two LTR circle forms of viral DNA were generated from doubling dilutions of HIV-1-infected CD4$^+$ MT-4 cells. Tubulin served as an internal cellular standard for all samples assayed; *, numbers represent cell equivalents.

TABLE 1. Quantitative effects of IFN-α treatment on HIV-1 reverse transcripts[a]

MOI-1	Viral DNA copy no. (fold reduction) for HIV-1 strain and IFN-α dose							
	$HIV\text{-}1_{ADA}$				$HIV\text{-}1_{DJV}$			
	0	50	500	5,000	0	50	500	5,000
LTR U3/R (early), 10^3	58.4	19.6 (2.9)	18.6 (3.1)	14.1 (4)	50.5	37.5 (1.3)	31.5 (1.6)	21.4 (2.3)
polI/J (intermediate)	518	323 (1.6)	190 (2.7)	107 (4.8)	262	143 (1.8)	76 (3.4)	27 (9.7)
LTR R/*gag* (late)	1,746	1,007 (1.7)	636 (2.7)	392 (4.4)	802	128 (6.2)	44 (18.2)	22 (36.4)
LTR R/U5 (2 LTR circles), 10^3	185	34 (5)	11.9 (15.5)	3 (61)	244	63 (3.9)	3 (81)	3 (81)

[a] Hybridized blots were visualized on a molecular Phosphoimager SF (Molecular Dynamics). To generate copy numbers, total counts were obtained from the 48-h time point of the hybridized blots and compared with known standards for LTR U3/R, *polI/J*, and LTR R/*gag* products generated by PCR on doubling dilutions of DNA from 8E5 cells (9). Linear curves were established from the 8E5 cells to generate the copy numbers shown. Standards for the two LTR circle forms of viral DNA were generated from doubling dilutions of HIV-1-infected $CD4^+$ MT-4 cells. Doses are measured in international units per milliliter.

quantitatively reduced and dependent on the IFN-α concentration. These results, taken together, demonstrate that IFN treatment of monocytes quantitatively diminishes the synthesis of all products of reverse transcription while preferentially affecting intermediate and late viral cDNA synthesis. The data are in agreement with our previous observations regarding the antiviral effects of IFN-α, in monocytes, including but not limited to the level of HIV-1 reverse transcription (1, 13).

IFN-induced restriction of the early events of the viral replication cycle in murine and avian retroviral infections, resulting in reduced efficiency of host cell transformation, have already been reported (31). Previous studies with HIV-1 demonstrated that IFN inhibits early and terminal events of viral replication, including nucleic acid synthesis, assembly, and budding of progeny virions, resulting in abortive infections or the formation of defective particles attached to host cell membranes (1, 10, 12, 13, 17, 19, 26, 30–34). In the present study, both early (viral cDNA synthesis) and late (progeny virion production) events of viral replication were analyzed and indicated that the reduction in virus production could not be accounted for solely by the observed reductions in viral DNA synthesis. This work is in agreement with previous published observations (26, 32) which indicate that IFN-α inhibition of HIV-1 replication is manifest predominantly at the level of viral cDNA synthesis. However, data presented in this study strongly support the notion that antiviral actions of IFN-α extend beyond the inhibition of RT. The use of single-cycle infections to measure viral nucleic acid synthesis and its nuclear transport coupled with measurements of spreading viral infections verify IFN-α's ability to restrict HIV-1 replication beyond viral cDNA synthesis. The importance of this work is that it reconciles, in part, the seemingly disparate reports (1, 10, 12, 13, 17, 19, 26, 30–34) concerning IFN-α's antiviral actions in HIV-1-infected cells.

The use of monocytes as indicator cells in laboratory assays provides a unique model for IFN-α antiretroviral actions. IFN-α markedly restricts HIV-1 replication in monocytes at levels sometimes exceeding those in $CD4^+$ T cells (13, 19, 26, 30). IFN-α is produced, in large measure, by monocytes (10). Most importantly, monocytes and macrophages are a major tissue reservoir for HIV-1 in the infected human host (14). There remains little doubt that the ability of IFN-α to restrict viral replication in macrophages has potential importance for treatment strategies in infected individuals (17). Virus-host cell interactions are unique in macrophages (16, 21, 29), since they are dependent not only on the viral strain (16, 21, 29) but on host cell differentiation and/or maturation (15, 27) and cytokines produced as a consequence of HIV-1 infection (7). Indeed, IFN-α and -β are regulated by cell-to-cell interactions between HIV-1-infected monocytes or T cells and peripheral blood mononuclear cells or by direct effects of the viral envelope protein gp120 (12, 18). Early events of viral replication (during or before acute seroconversion) may be controlled, in part, by IFN (28). Only later during advanced viral infection and immunosuppression do HIV-1 mutants with apparent IFN resistance phenotypes likely emerge (20, 30a). The presence of IFN resistance phenotypes of HIV-1 could circumvent antiviral actions of IFN. At the same time, these viruses could retain the ability to induce IFN within infected individ-

TABLE 2. Quantitative effects of IFN-α treatment on HIV-1 reverse transcripts[a]

MOI-1	Viral DNA copy no. (fold reduction) for HIV-1 strain and IFN-α dose							
	$HIV\text{-}1_{ADA}$				$HIV\text{-}1_{DJV}$			
	0	50	500	5,000	0	50	500	5,000
LTR U3/R (early), 10^3	20.9	1 (20.9)	3.3 (6.3)	3.7 (5.6)	30.8	12.9 (2.4)	8.9 (3.5)	8.7 (3.5)
polI/J (intermediate)	344	28 (12.2)	21 (16.3)	8 (43)	146	37 (3.9)	10 (14.6)	5 (29.2)
LTR R/*gag* (late)	1,444	286 (5)	164 (8.8)	77 (18.7)	135	19 (7)	4 (33.7)	0
LTR R/U5 (2 LTR circles), 10^3	50	20 (2.5)	0	0	16	0	0	0

[a] Hybridized blots were visualized on a molecular Phosphoimager SF (Molecular Dynamics). To generate copy numbers, total counts were obtained from the 48-h time point of the hybridized blots and compared with known standards for LTR U3/R, *polI/J*, and LTR R/*gag* products generated by PCR on doubling dilutions of DNA from 8E5 cells (9). Linear curves were established from the 8E5 cells to generate the copy numbers shown. Standards for the two LTR circle forms of viral DNA were generated from doubling dilutions of HIV-1-infected $CD4^+$ MT-4 cells. Doses are measured in international units per milliliter.

uals. The inevitable outcome of this virus-macrophage-cytokine interaction is high levels of plasma viremia coordinate with high titers of IFN-α and leading to advanced clinical disease (17, 30).

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