Interferon γ Induces the Expression of Human Immunodeficiency Virus in Persistently Infected Promonocytic Cells (U1) and Redirects the Production of Virions to Intracytoplasmic Vacuoles in Phorbol Myristate Acetate-differentiated U1 Cells

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Summary

Interferon γ (IFN-γ), a lymphokine that exerts multiple immunoregulatory effects, has been found to be elevated in the plasma, cerebrospinal fluid, and lymph nodes of human immunodeficiency virus (HIV)-infected individuals and has shown variable effects on HIV replication in acutely infected cells. In the present study, we have demonstrated that IFN-γ is a potent modulator of HIV expression in persistently infected U1 promonocytic cells in which virus production is characterized by a constitutive state of relative latency. Direct stimulation of U1 cells with IFN-γ (10–1,000 U/ml) activated HIV expression, as measured by reverse transcriptase (RT) activity in the culture supernatant and increased levels of cell-associated viral protein and mRNAs. These effects on virus expression were not accounted for by the induction of endogenous TNF-α secretion, as previously described in U1 cells stimulated with phorbol myristate acetate (PMA). At the ultrastructural level, the stimulatory activity of IFN-γ was correlated with HIV particle production in intracytoplasmic vacuoles along with the differentiation of U1 into macrophage-like cells. Furthermore, costimulation of U1 cells with IFN-γ and PMA significantly increased the accumulation of vacuole-associated HIV concomitant with decreasing membrane-associated particles and RT activity production, as compared with cells stimulated with PMA alone. No evidence of spontaneous secretion of intracellular vacuole-associated virus was obtained by kinetic analysis of the RT activity released in the supernatants throughout the culture period unless cells were deliberately disrupted. These findings suggest that vacuole-associated virions likely represent a relatively stable intracellular reservoir of HIV, as previously described in primary macrophages infected in vitro or in infected macrophages in the brains of patients with acquired immune deficiency syndrome. The reduced levels of RT activity observed in the culture supernatants of U1 cells stimulated with PMA in the presence of IFN-γ were not indicative of a suppressive effect of IFN-γ on PMA-induced expression of HIV proteins and mRNAs, either directly or mediated by the release of IFN-α/β. This study suggests that IFN-γ may play an important role as an inducer of HIV expression in infected mononuclear phagocytes.

IFN-γ is a potent lymphokine produced by activated T cells that mediates several immunomodulatory and antimicrobial effects (reviewed in references 1–4). Both in vivo and in vitro studies have previously investigated the potential role of IFN-γ in HIV infection. Elevated levels of IFN-γ and/or of the IFN-γ-inducible factor neopterin have been found in the plasma and cerebrospinal fluid (CSF)[1] of HIV-infected individuals at various stages of disease (5–7). In contrast, PBMC from HIV-

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[1] Abbreviations used in this paper: CSF, cerebrospinal fluid; MDM, monocyte-derived macrophages; MP, mononuclear phagocytes; RT, reverse transcriptase.

infected individuals are usually characterized by a significantly impaired ability to generate IFN-γ in vitro (8–10), although purified subpopulations of T cells showed an intact capacity to secrete this lymphokine (11). High levels of IFN-γ RNA were recently demonstrated in the lymph nodes of HIV-infected individuals (12), suggesting that this lymphokine may influence the ability of HIV to replicate in CD4⁺ T lymphocytes and mononuclear phagocytes (MP) in vivo.

In vitro, IFN-α and IFN-β, but not IFN-γ, inhibited HIV replication in mitogen-stimulated PBMC (13) in which IFN-γ may actually act as an endogenous autocrine/paracrine inducer of HIV replication in conjunction with TNF-α/β (14). In other studies, the combination of IFN-γ and TNF-α has been reported to induce cytolysis of HIV-infected cell lines but not of their uninfected counterparts, in association with a reduction of viral RNA (15). In addition, IFN-γ showed suppressive effects on HIV replication in certain T cell lines (16) and in the promonocytic cell line U937 (17, 18), which were acutely infected with HIV. In primary human monocyte-derived macrophages (MDM), IFN-α, -β, and -γ suppressed HIV replication when added to culture either before or up to 3 d after infection (19). However, other investigators have observed dichotomous effects of IFN-γ on virus replication in MDM, in that inhibition of virus expression was seen in cells pretreated with this lymphokine, whereas upregulation of p24 Ag production occurred if cells were treated with IFN-γ after infection (20). These studies suggest that IFN-γ may play different and even opposite roles in the regulation of HIV replication in different cell types. However, little or no information is available on the effects of IFN-γ in cells persistently infected with HIV. In this regard, several in vivo and in vitro studies have underscored the importance of persistent HIV infection in the pathogenesis of AIDS (21). Quantitative studies have demonstrated that in the peripheral blood compartment the number of infected cells that are in a state of either relative or absolute viral latency exceeds by about 10-fold the number of cells actively expressing HIV RNA or proteins (22). Most of these circulating infected cells are CD4⁺ T lymphocytes, whereas monocytes in the peripheral blood are infrequent targets of HIV infection in vivo (22–24). In contrast, terminally differentiated macrophages represent a major viral reservoir in several tissues and organs, including the brain (25, 26) and the lungs (27) of HIV-infected individuals, and have been found to express high levels of viral RNA (25, 26). One distinctive aspect of HIV infection in macrophages is the ability of the virus to bud from and accumulate into intracellular vacuolar compartments (28–32), an uncommon feature in infected T lymphocytes. This observation, coupled with the lower susceptibility of MP to the cytocidal effects of HIV (23, 28), has suggested that tissue macrophages may represent important viral reservoirs that play a major role in the establishment of a state of chronic infection typical of most infected individuals (21, 32). Therefore, understanding whether physiologic host factors can influence the ability of HIV to assemble and accumulate in intracellular compartments as opposed to the plasma membrane of infected macrophages may be of importance for

designing therapeutic strategies targeting HIV at its different cellular and sub-cellular sites of expression. In this regard, it has been reported that IFN-α effectively suppressed the release of HIV particles from the plasma membrane of chronically infected T lymphocytic cells (33–35), a phenomenon that had been already described in cells persistently infected with other retroviruses after treatment with IFNs (36).

In the present study, we have investigated the effect of IFN-γ on the chronically infected promonocytic cell line U1, in which virus expression is modulated by several cytokines and pharmacologic agents (reviewed in references 21 and 37). PMA stimulation of U1 cells not only induces HIV production, but also induces the terminal differentiation of these cells along the mononuclear phagocytic lineage in association with the accumulation of HIV particles in Golgi-derived intracellular vacuoles (38), as described in primary infected macrophages (28–32). Thus, we determined the effects of INF-γ alone or in costimulation with PMA on cellular morphology, virus production, and virion compartmentalization in U1 cells.

Materials and Materials

Chronically HIV-infected Cell Lines. The U1 cell line was established from a population of U937 promonocytic cells surviving an acute infection with HIV-1, as described previously in detail (38). U1 cells contain two integrated copies of HIV proviral DNA, and are characterized by low levels of constitutive virus expression that can be modulated by several cytokines and pharmacologic agents (21, 37–40). 1–2 × 10⁵ U1 cells/ml were resuspended in RPMI 1640 (M.A. Whitaker Bioproducts, Walkersville, MD) containing 10% FCS, plated in 96-well flat-bottomed plates (Costar, Cambridge, MA), and incubated with either rIFN-γ (Genzyme, Boston, MA), rIFN-α₂ᴮ (Schering, Kenilworth, NJ), rIFN-β (kind gift of Dr. Sidney Petska, UMDNJ, Robert Wood Johnson Medical School, Piscataway, NJ), PMA (10⁻⁷ to 10⁻⁸ M) (Sigma Chemical Co., St. Louis, MO), rTNF-α, or rTNF-β (100 U/ml) (Genzyme), at 37°C in 5% CO₂. Supernatants from the cell cultures were collected at various times after stimulation and stored at −70°C until tested for the presence of reverse transcriptase (RT) activity.

RT Activity Assay. 5 μl of culture supernatants was added in duplicate or triplicate to 25 μl of a mixture containing poly(A), oligo(dT) (Pharmacia Fine Chemicals, Piscataway, NJ), MgCl₂, and [³²P]-labeled deoxythymidine 5'-triphosphate (dTTP) (Amersham Corp., Arlington Heights, IL), and incubated for 2 h at 37°C. 6 μl of the mixture was spotted onto DE81 paper, air-dried, washed five times in 2× SSC buffer, and two additional times in 95% ethanol. The paper was then dried, cut, and counted on a scintillation counter (LS 7000; Beckman Instruments, Inc., Fullerton, CA). Variability of replicate cultures was always <15%.

Western Blot Analysis of Cell-associated HIV Proteins. Lysates were prepared from U1 cells either unstimulated or stimulated for 48 h with IFN-γ (1,000 U/ml), PMA (10⁻⁸ M), or costimulated with IFN-γ plus PMA. 20 μl from the lysate of 10⁷ cells was added to each lane and subjected to electrophoresis through 10–20% gradient polyacrylamide gels (Integration Sep. Sci., Hyde Park, MA) for 6 h. The migrated proteins were then transferred overnight onto nitrocellulose filters. After saturation with a 5% milk solution, filters were incubated for 2 h with 1:1,000 (vol/vol) dilution of an AIDS patient serum containing high titers of anti-HIV Ab recognizing most of the major viral proteins (33). Filters were then washed

and incubated for 90 min with ^{125}I labeled protein A (200,000 dpm/ml), washed, air-dried, and exposed overnight to x-ray film.

Analysis of HIV RNA. U1 cells were either unstimulated, or stimulated for 24 h with IFN-γ (1,000 U/ml), TNF-α (100 U/ml), and PMA (10⁻⁷ M), or costimulated with PMA plus IFN-γ. Total RNA was extracted from 2×10^7 U1 cells by the guanidine thiocyanate phenol method using an RNA isolation kit (Stratagene, La Jolla, CA). In some experiments, different amounts of RNA were transferred to nitrocellulose filters in an RNA slot blot Minifold II apparatus (Schleicher & Schuell, Inc., Keene, NH), and the filters were hybridized overnight with a ³²P labeled 6.4-kb DNA probe (pBenn 5; map position, 1.7–8.1 kb), washed, and exposed to x-ray film as described (41). For Northern blot analysis, 10 μg of total RNA extracted at different times after stimulation was loaded per lane on a 0.8% agarose formaldehyde gel and transferred to nitrocellulose. The filters were baked and hybridized for 12 h with a ³²P labeled HIV-LTR homologous probe (SST-BssHII). Filters were washed and exposed to x-ray film. The labeled probe was removed from the filters by washing at 80°C in 0.1× SSC containing 0.1% SDS; the filters were then rehybridized with a ³²P labeled β-actin cDNA probe, as previously described (42).

Ultrastructural Studies. U1 cells were stimulated with either IFN-α (20–100 U/ml), IFN-β (100 U/ml), IFN-γ (100–1,000 U/ml), PMA (10⁻⁸ M), or costimulated with IFN-α and PMA, IFN-β and PMA, or IFN-γ and PMA for 36–120 h. Cells were spun, fixed in glutaraldehyde, and then postfixed in OsO₄, dehydrated in graded ethanol and propylene oxide, and embedded in plastic, as described (33). For each sample, 100 consecutive cell sections were screened for the presence and location of HIV. Only sections at or close to the center of the cell and Golgi compartment (based on known average size of cell and nucleus) were included for evaluation. The findings were tallied as percentage of cells with HIV budding from or intimately associated with the plasma membrane only, HIV present in cytoplasmic vacuoles only, or HIV observed at both sites in the same cell.

Results

IFN-γ Induces HIV Expression in Chronically Infected U1 Cells. Stimulation of U1 cells with different concentrations of IFN-γ (10–1,000 U/ml) induced HIV production, as measured by RT activity in the culture supernatants, although to a lesser extent than did PMA stimulation (Fig. 1). Higher concentrations of IFN-γ (up to 5,000 U/ml) did not result in a significant increase of virus production as compared with the levels of induction observed with 500–1,000 U/ml of this lymphokine (data not shown). Thus, IFN-γ, which has been previously reported to induce HIV expression in acutely infected primary MDM (20), is another in the group of cytokines capable of inducing virus expression in persistently infected monocytic cells (reviewed in reference 37). In this regard, cytokines that stimulate HIV expression may induce de novo transcription of viral RNAs, as demonstrated for TNF-α and TNF-β (21, 37, 39–42), or may predominantly affect posttranscriptional steps in the HIV life cycle, as shown for IL-6 (42). We therefore investigated the effect of IFN-γ on the expression of HIV RNA in U1 cells. Unstimulated U1 cells showed detectable levels of fully spliced 2-kb mRNA of the regulatory genes *tat*, *nef*, and *rev* (Fig. 2), as previously reported (40). Stimulation of U1 cells with IFN-γ resulted in the ac-
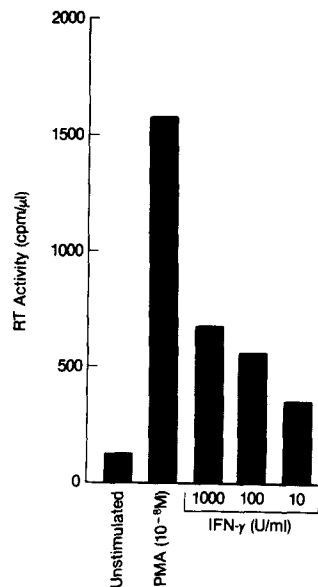

Figure 1. IFN-γ induces HIV expression in U1 cells. U1 cells were cultured in either unstimulated conditions or with various concentrations of IFN-γ. Culture supernatants were collected after 72 h stimulation and tested for the presence of RT activity. The results are the average of duplicate samples per each culture condition and are representative of at least 10 separate experiments. Similar results were obtained by determination of the levels of HIV p24 Ag in the culture supernatants, using a commercially available ELISA kit (Coulter Electronics, Hialeah, FL).

cumulation of all three species of HIV mRNA, including high levels of the unspliced 9-kb mRNA coding for gag-pol structural protein (Fig. 2), suggesting that IFN-γ may have a direct inductive effect on HIV transcription in monocytic cells, albeit with delayed kinetics as compared with TNF-α (Fig. 2) or PMA (data not shown).
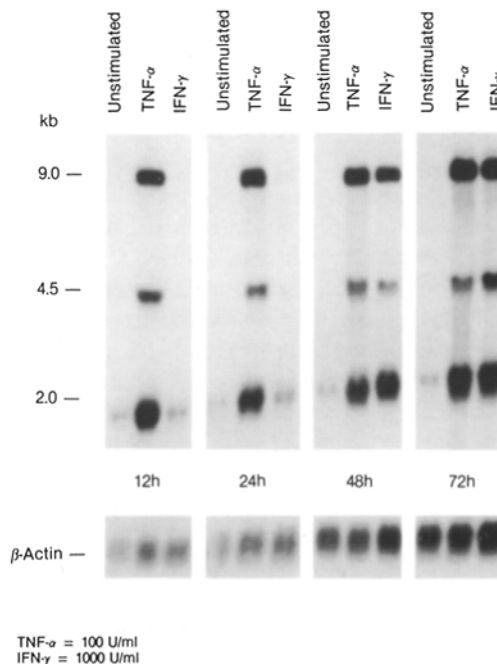

TNF-α = 100 U/ml
IFN-γ = 1000 U/ml

Figure 2. IFN-γ induces the accumulation of both spliced and unspliced HIV mRNAs. Total RNA was extracted from unstimulated U1 cells or from cells stimulated with either IFN-γ (1,000 U/ml) or TNF-α (100 U/ml) after 12, 24, 48, and 72 h of incubation, as described. IFN-γ induced significant levels of HIV RNA, although with delayed kinetics compared with TNF-α-stimulated cells.

The Upregulatory Effect of IFN-γ on HIV Expression Is Not Mediated by the Production of Endogenous TNF-α or TNF-β. The observation that the induction of steady-state HIV mRNA by IFN-γ had delayed kinetics as compared with TNF-α or PMA suggested the possibility that the effect of IFN-γ could be mediated by the production of a second cellular factor. In this regard, we have previously reported that stimulation of U1 cells with PMA resulted in the synthesis and release of TNF-α, which actually mediated most of PMA-induced virus production (43). Therefore, we investigated the possibility that stimulation of U1 cells with IFN-γ, similar to PMA, triggered an autocrine pathway of viral induction mediated by endogenous TNF-α or TNF-β; this possibility was also supported by the well-described ability of IFN-γ to induce TNF production in different cell types (reviewed in references 1-4), including MP (44). However, in contrast to cells stimulated with PMA, no evidence of TNF-α secretion (Fig. 3 A) and/or gene transcription (data not shown) were obtained in U1 cells stimulated with IFN-γ. Anti-TNF-α and anti-TNF-β Ab completely suppressed the induction of HIV expression mediated by their respective cytokine, but did not affect the ability of IFN-γ to induce HIV expression in U1 cells (Fig. 3 B). In addition, an anti-TNF-α mAb that significantly reduced the accumulation of HIV mRNAs in both TNF-α-stimulated and PMA-stimulated U1 cells did not affect IFN-γ induction of viral messages (data not shown).

Ultrastructural Studies of HIV Particle Synthesis in U1 Cells Stimulated with IFN-γ in the Absence and Presence of PMA. IFN-γ is a well-known activator of MP function (1–3). In addition, this lymphokine is known to exert profound effects on the differentiation of MP, including immature elements such as U937 promonocytic cells (the parental uninfected cell line of U1) (44–46). Because similar or even more profound effects on phagocyte activation and differentiation, as well as HIV expression, have been described after treatment with phorbol esters (46–48), we compared IFN-γ and PMA with regard to the influence of their cellular differentiative effects on the process of virion maturation in U1 cells. In this regard, we have previously described that PMA stimulation of U1 cells results in the rapid acquisition of the morphological (increased size, irregular plasma membrane, eccentric nucleus, prominent Golgi compartment, increased number of lysosomes) and functional (adherence to plastic surface, ability to secrete cytokines) characteristics of differentiated macrophages (38, 43). In addition, PMA stimulation of U1 cells induced budding and release of virions both at the plasma membrane as well as in Golgi-derived intracytoplasmic vacuoles, resembling effects independently described in primary human macrophages infected with HIV in vitro (28–30) or in vivo (31). IFN-γ stimulation of U1 cells resulted in distinct features of cell differentiation along the MP lineage similar to those observed after PMA treatment (Fig. 4, A and B). Furthermore, HIV particle production after IFN-γ stimulation was demonstrated both at the plasma membrane (Fig. 4, B and C) as well as in intracytoplasmic vacuoles (Fig. 4, B and D). However, these features were clearly observed only in a fraction (<10%) of U1 cells stimulated with IFN-γ. This
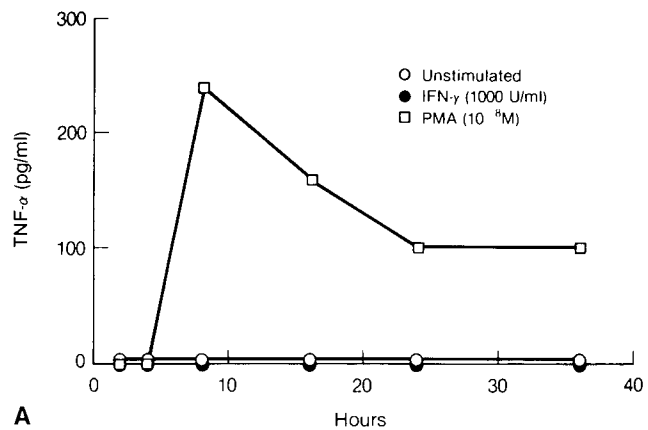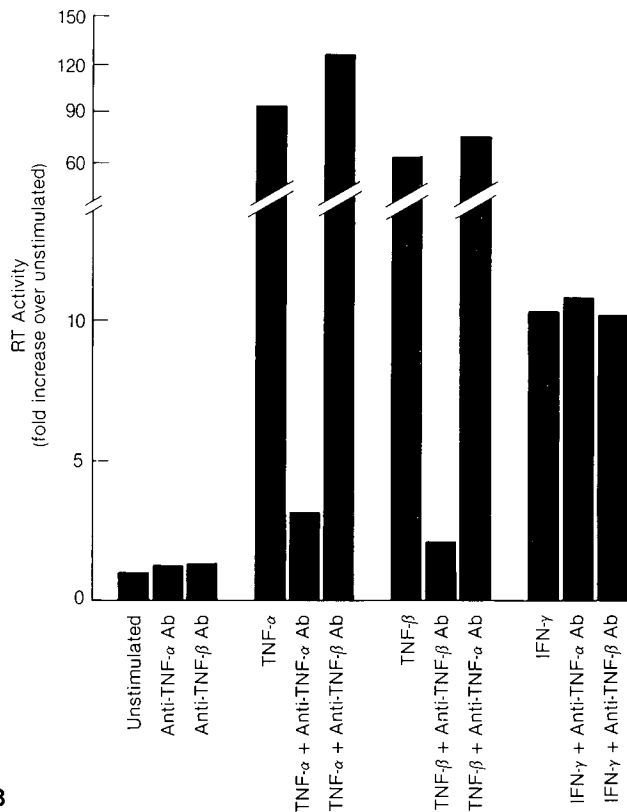
Figure 3. IFN-γ induction of HIV expression is not mediated by the secretion of endogenous TNF-α. (A) PMA stimulation of U1 cells induced the secretion of detectable levels of TNF-α, whereas no significant levels of this cytokine were observed in either unstimulated cells or cells stimulated with IFN-γ. Furthermore, IFN-γ did not inhibit PMA-induced secretion of TNF-α (not shown). The levels of TNF-α present in the culture supernatants were determined by the use of an ELISA kit (Quantikine; R & D Systems, Minneapolis, MN). (B) Effect of anti-TNF-α mAb (Cutter Biological, Miles Inc., Berkeley, CA) and anti-TNF-β-neutralizing Ab (R & D Systems) on HIV expression in U1 cells. Anti-TNF-α mAb (1 μg/ml) and anti-TNF-β polyclonal Ab (50 μg/ml) completely neutralized the inductive effect of TNF-α or TNF-β on HIV expression, respectively, without affecting IFN-γ-mediated upregulation of RT activity.

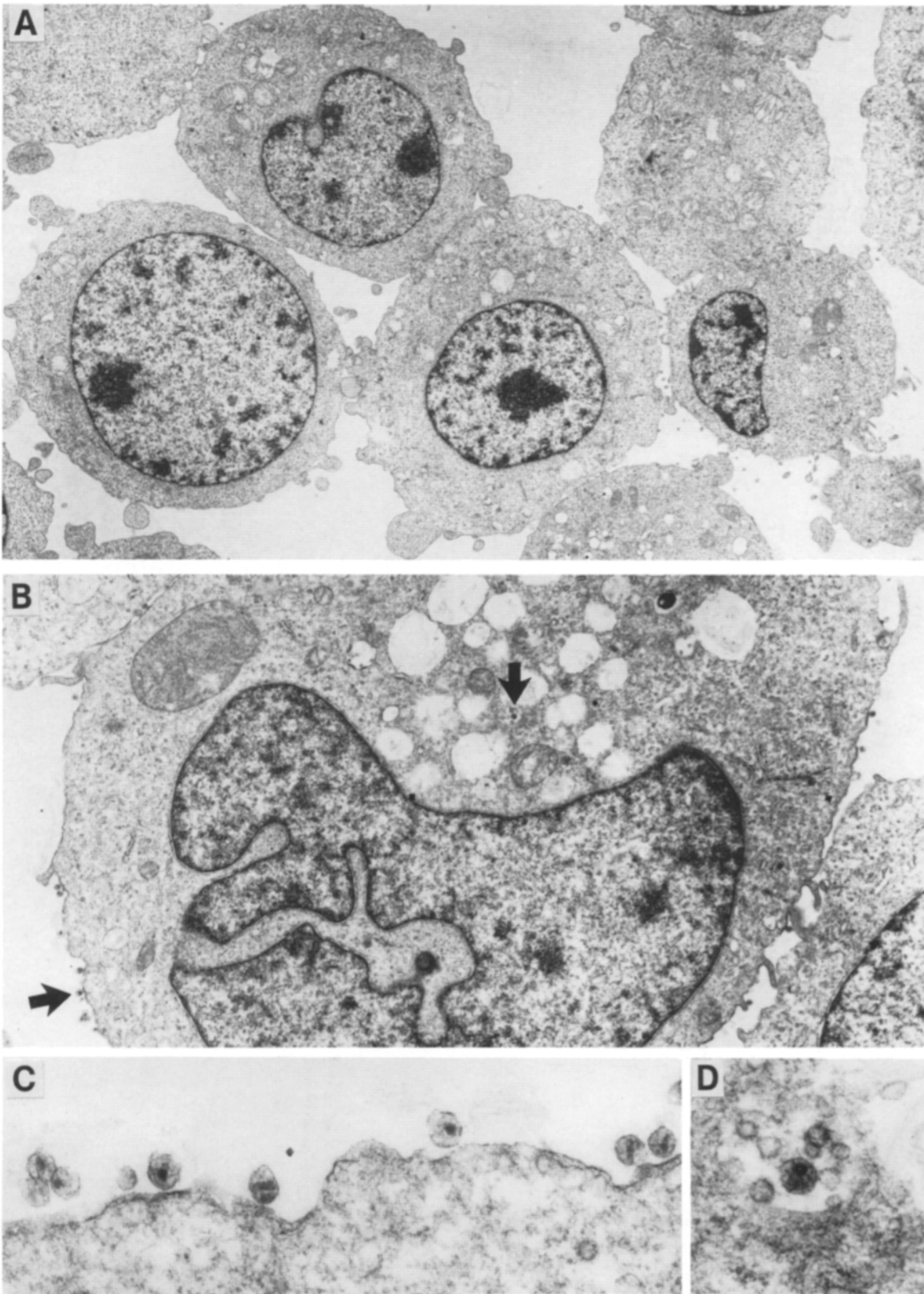**Figure 4.** IFN-γ induces HIV particle expression and U1 cell differentiation. (*A*) Transmission electron microscopy of unstimulated U1 cells. The cytoplasm is dominated by ribosomes with only scattered mitochondria and profiles of rough endoplasmic reticulum and inconspicuous Golgi zones. (*B*) A portion of a U1 cell stimulated with IFN-γ for 72 h. Small clusters of virions are seen at several locations on the plasma membrane (*arrow* point to one cluster on bottom left, enlarged in *C*). The Golgi region is filled with vacuoles, one of which contains a mature virion (*arrow*, enlarged in *D*). (*A*) ×4,200; (*B*) ×10,000; (*C*) ×49,000; (*D*) ×66,000.
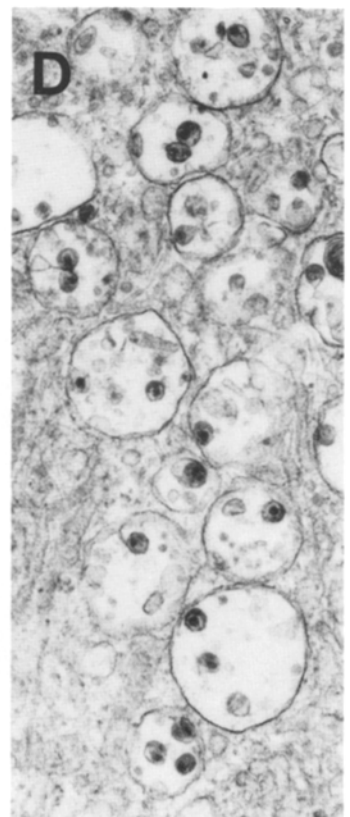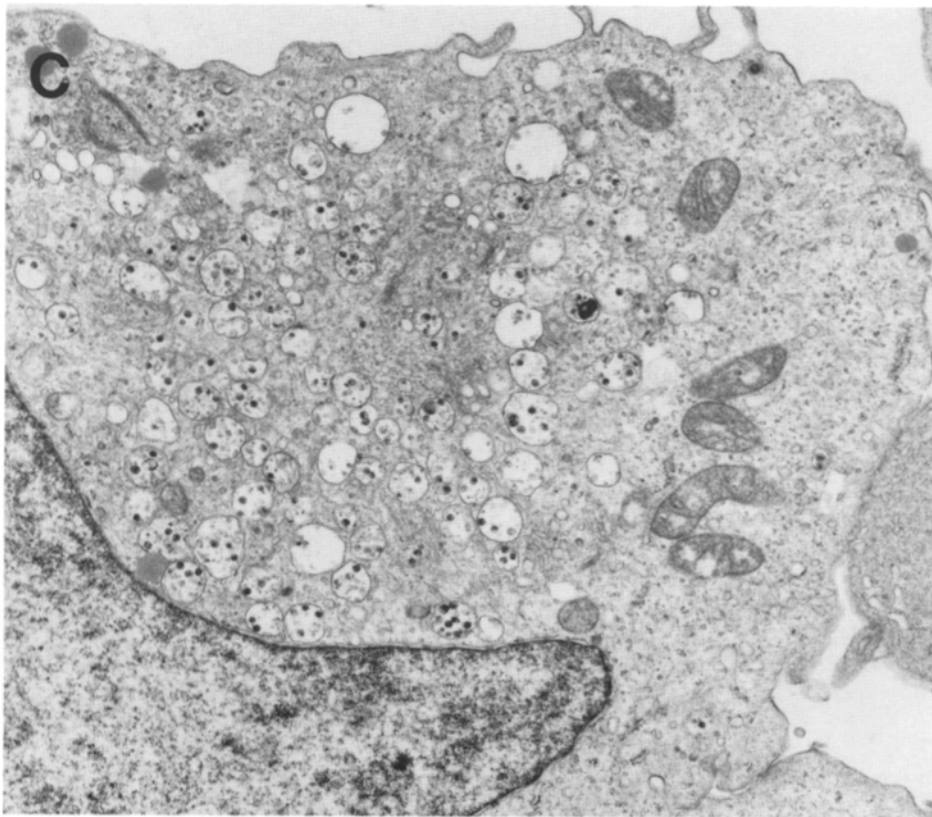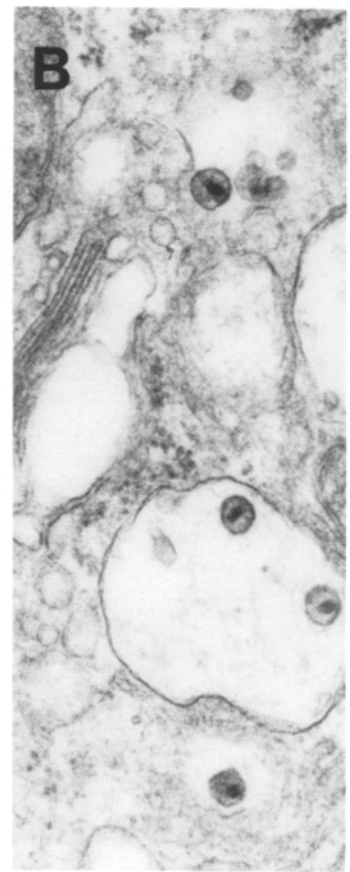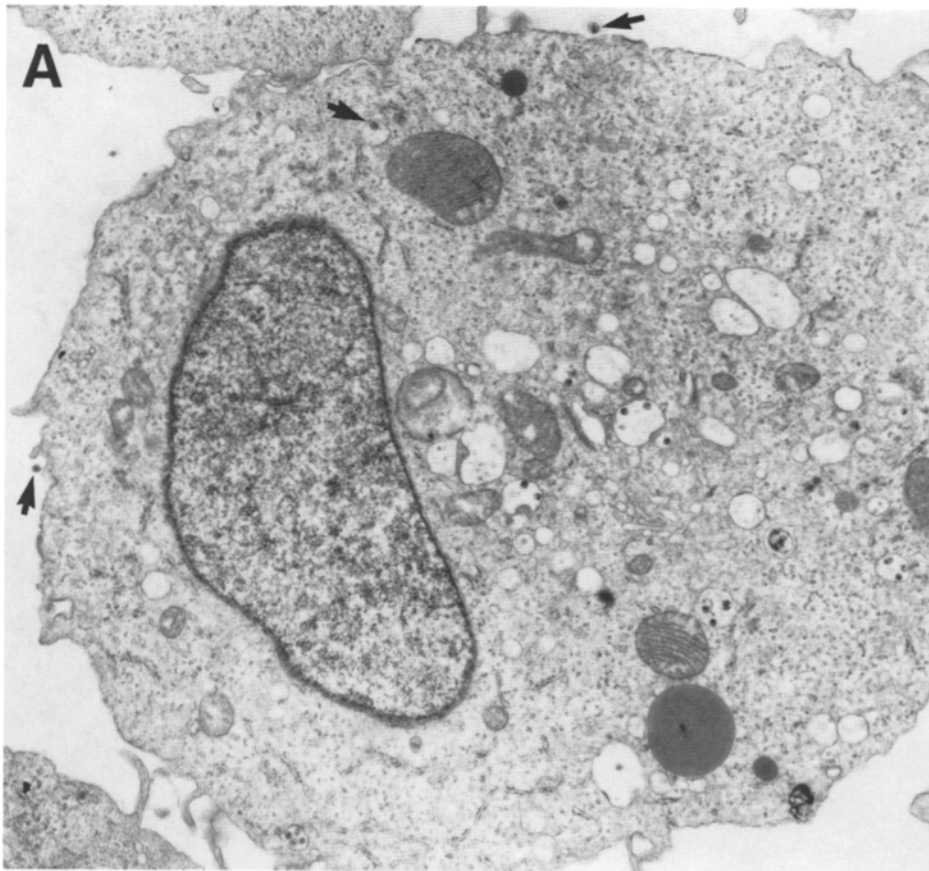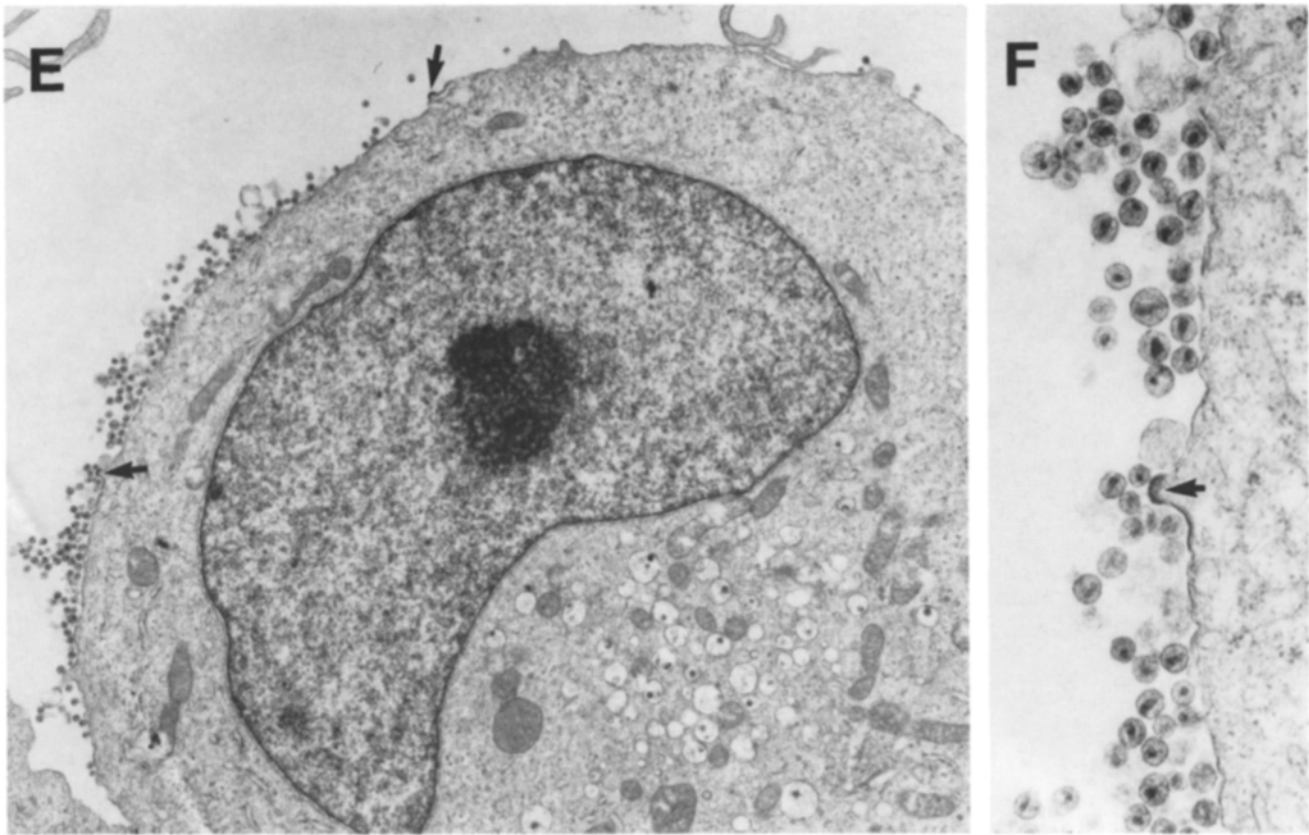
Figure 5. Effect of IFN-γ and IFN-α on PMA stimulated U1 cells. U1 cells were stimulated with PMA (10^{-8} M) either in the presence or absence of IFN-γ (1,000 U/ml) or IFN-α (100 U/ml) for 48 h. Cells were then fixed and analyzed by transmission electron microscopy. (*A* and *B*) PMA stimulation of U1 cells induced viral particle production both at the plasma membrane as well as in intracytoplasmic compartments. As compared with the unstimulated U1 cells (Fig. 4 *A*), PMA stimulation causes the cell to become more irregular in outline, with an eccentric indented nucleus, and a more prominent Golgi region. Small numbers of typical HIV particles are seen associated with the plasma membrane (*arrows*) and within Golgi-derived cytoplasmic vacuoles, some of which are enlarged in *B*. (*C* and *D*) IFN-γ decreased the levels of plasma membrane–associated virus, but consistently increased the intracellular accumulation of virions in cells stimulated with PMA. The Golgi region is gently expanded by vacuoles containing HIV (several enlarged in *D*). No HIV particles were detected on the surface of this cell in this section. (*E* and *F*) IFN-α caused both the accumulation of virions in intracellular vacuoles as well as their aggregation on the plasma membrane. A stretch of the plasma membrane is covered by multiple layers of typical HIV particles, at least two of which are in the budding stage (*arrows*). The Golgi region (lower right of *E*) is expanded by many vacuoles. (*A*) ×9,400; (*B*) ×39,000; (*C*) ×9,700; (*D*) ×32,000; (*E*) ×,8,400; (*F*) ×32,000.

is different from stimulation of U1 with PMA in which similar morphological and functional changes were seen in the majority of the cells. We therefore investigated the ability of IFN-γ to modulate virus production in cells stimulated with PMA. In this regard, PMA and IFN-γ have been described as exerting synergistic effects on a variety of functions of MP cells, including the U937 cell line (45, 49). After 48 h of PMA stimulation, ~50–85% of U1 cells expressed typical HIV particles at the plasma membrane and/or in intracytoplasmic compartments (Fig. 5, *A* and *B*) compared with unstimulated cells in which virion production was essentially undetectable (Fig. 4 *A*). When U1 cells were stimulated with both PMA and IFN-γ, no changes in terms of the total number of cells expressing virus was observed. However, costimulation with PMA and IFN-γ compared with stimulation with PMA alone caused a threefold decrease in the number of cells expressing HIV exclusively at the plasma

membrane level, and a concomitant fourfold increase in cells in which particles were detected only in intracytoplasmic vacuoles (Figs. 5, *C* and *D*, and 6 *A*). This effect was demonstrated both in terms of a shift in the proportion of cells with intravacuolar virus as well as in the total number of virions present within the vacuoles (Fig. 5, *C* and *D*).

IFN-γ-mediated Accumulation of Virions in Intracytoplasmic Vacuoles Results in Decreased HIV Production in PMA-stimulated U1 Cells. Having established that costimulation of U1 cells with PMA and IFN-γ induced a redirection of the preferential site of virus expression from the plasma membrane to intracytoplasmic vacuoles, we next investigated the kinetics of this phenomenon. A progressive increase in the levels of vacuole-associated HIV particles was observed up to 5 d after U1 cell stimulation with either PMA or PMA plus IFN-γ (in which cultures this phenomenon was significantly more pronounced) (Fig. 6 *B*). In contrast, a reduction of plasma-
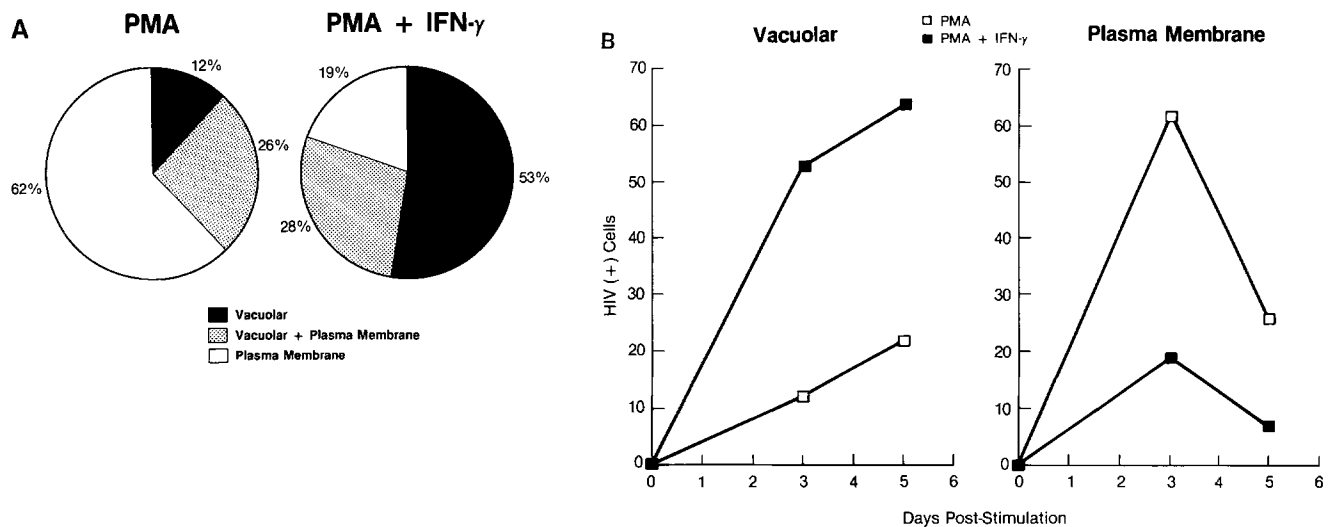
Figure 6. IFN-γ increases the intracellular accumulation of HIV particles in PMA-stimulated U1 cells. (*A*) A relative increase in the percentage of cells exclusively showing virions budding from and contained within intracytoplasmic vacuoles was observed in cells costimulated with PMA and IFN-γ compared with cells stimulated with PMA alone, as shown in Fig. 5. Results were quantified by transmission electron microscopy after 72 h of stimulation. The results are representative of three independent experiments. (*B*) Kinetics of virion accumulation at the plasma membrane and in intracytoplasmic vacuoles. The results are obtained by the analysis of the morphological features of 100 consecutive cells from one representative experiment out of three independently conducted. The simultaneous expression of virions in vacuoles and at the plasma membrane (not shown) was relatively stable in that it was seen in ∼30% of U1 cells stimulated with either PMA alone or with PMA plus IFN-γ both after 3 and 5 d of culture.

membrane associated virus was seen in cells stimulated with PMA in the presence of IFN-γ as compared with cells induced by PMA alone (Fig. 6 *B*). Furthermore, from a kinetic standpoint, virus tended to accumulate over time in the vacuolar compartment as opposed to the plasma membrane whether cells were stimulated with PMA alone or with PMA plus IFN-γ. This was underscored by the observation that lower levels of HIV were exclusively associated with the plasma membrane after 5 d compared with 3 d of culture regardless of the stimuli, while the levels of virions segregated exclusively in vacuoles increased from day 3 to day 5 in culture (Fig. 6 *B*). We further investigated whether these morphological changes had a functional impact on the ultimate release of HIV from the infected cells. In this regard, we have previously confirmed that measurement of RT activity production from U1 cells (as well as other cell lines persistently infected with HIV) is a valid parameter of HIV particle release, in that no detectable RT activity was demonstrable in column fractions containing particle-free viral proteins (such as p24 Ag) shed from infected cells (50). A significant decrease of the RT activity levels released by U1 cells stimulated with PMA in the presence of IFN-γ was observed compared with cells stimulated with PMA alone (Fig. 7), suggesting that the IFN-γ-mediated redirection of virus production within intracytoplasmic vacuoles resulted in a relatively stable intracellular sequestration of virions throughout the culture period.

IFN-γ Does Not Suppress the Synthesis of HIV mRNAs and Proteins in PMA-stimulated Cells. Although the ultrastructural analysis of U1 cells stimulated with PMA in the presence of IFN-γ did not indicate that this lymphokine had a suppressive effect on HIV biosynthesis, this technique allows one to examine only a small fraction of the total cell popula-

tion. We therefore investigated whether the reduced levels of RT activity observed in PMA-stimulated cells in the presence of IFN-γ could have been caused by reduced HIV mRNA and/or protein synthesis. No significant differences were observed between the levels of HIV mRNA in U1 cells stimu-
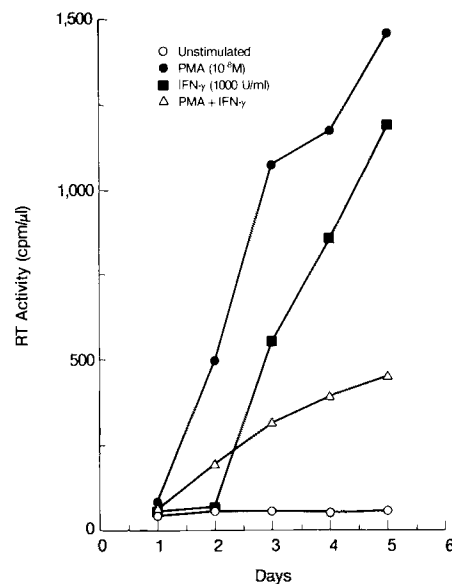

Figure 7. Suppressive effect of IFN-γ on PMA induction of RT activity in U1 cell. Kinetic analysis reveals that although IFN-γ alone induces RT activity in U1 cells, the stimulation with PMA and IFN-γ markedly suppresses the induction of RT activity compared with stimulation with PMA alone.

lated with PMA or PMA plus IFN-γ, as shown by quantitative slot-blot analysis (Fig. 8 A). Furthermore, no significant changes in the relative or absolute levels of spliced and unspliced HIV mRNAs were seen by Northern blot analysis of these cells (data not shown). A modest increase in the levels of cell-associated HIV proteins was observed in U1 cells stimulated with PMA plus IFN-γ as compared with cells activated with PMA alone, likely resulting from the independent inductive effect of these two agents (Fig. 8 B), and/or from the increased accumulation of vacuole-associated HIV particles in cells costimulated with PMA and IFN-γ (Figs. 5 and 6). Finally, cell disruption by multiple cycles of freezing and thawing resulted in the recovery of RT levels from U1 cells costimulated with PMA and IFN-γ to the levels of cells stimulated with PMA alone (data not shown), indicating that the reduced levels of supernatant-associated RT activity were not a consequence of decreased virus expression, but resulted from the accumulation of particles in intracellular compartments.

The Decrease in PMA-induced Virus Production in U1 Cells Stimulated with IFN-γ Is Not Mediated by Secretion of Endogenous IFN-α/β. We and others have previously described that IFN-α could suppress the production of RT activity in persistently infected T lymphocytic cell lines as a consequence of a block in the release of HIV particles from the plasma membrane and in the absence of a suppressive effect on HIV protein synthesis (33–35). To investigate whether the reduced production of HIV particles in the culture supernatant observed in cells stimulated with PMA in the presence of IFN-γ was a consequence of endogenously produced IFN-α, we compared the effect of IFN-α with that of IFN-γ in U1 cells stimulated with PMA at the ultrastructural level. Similar to IFN-γ, IFN-α increased the intracellular accumulation of virus particles in PMA-stimulated U1 cells (Fig. 5, E and F). How-

ever, unlike IFN-γ, which decreased the quantity of plasma membrane–associated virus, IFN-α caused the clustering of virions at the external plasma membrane (postbudding effect), as previously described in several infected T lymphocytic cell lines (33–35). Both IFN-α and IFN-γ inhibited PMA-induced virus expression, although IFN-α was consistently more potent in this regard than IFN-γ (Fig. 9). Anti-IFN-α Ab completely restored the levels of RT activity in the culture supernatants of cells stimulated with PMA plus IFN-α to the levels observed in cells stimulated with PMA alone, whereas it did not affect the suppressive effect of IFN-γ (Fig. 9). Similar results were observed in U1 cells incubated under these conditions for longer times, and in experiments using rIFN-β and anti-IFN-β-neutralizing Ab (data not shown), indicating that the suppressive effects of IFN-γ on RT production in PMA-stimulated U1 cells were not mediated by IFN-α or IFN-β. Of note is the fact that no inductive effects on HIV expression were seen in U1 cells treated with IFN-α alone (or IFN-β alone; data not shown), in contrast to cells stimulated with IFN-γ.

Discussion

In the present study we have demonstrated that IFN-γ is a direct inducer of HIV expression in persistently infected promonocytic U1 cells. The effect of IFN-γ was correlated
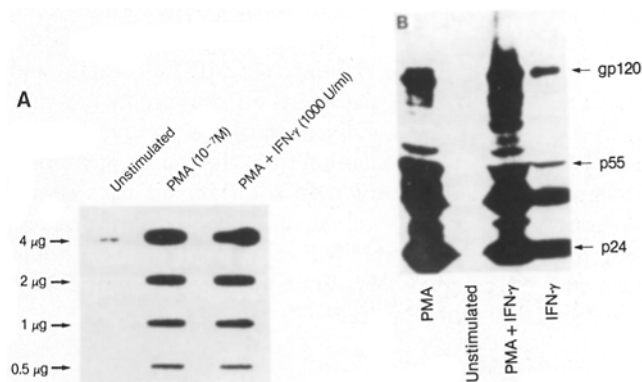

Figure 8. IFN-γ does not reduce the levels of PMA-induced HIV mRNA or proteins. (A) Slot-blot analysis of HIV RNA. Total RNA was extracted from either unstimulated U1 cells or from cells stimulated with PMA or PMA plus IFN-γ for 24 h, and was diluted in a slot-blot apparatus. Northern blot analysis confirmed that IFN-γ did not reduce PMA-induced accumulation of spliced and unspliced HIV mRNAs. (B) Direct induction of HIV protein synthesis in U1 cells stimulated with IFN-γ alone and lack of IFN-γ inhibition of PMA-induced viral proteins. The results represent the levels of cell-associated HIV proteins present after 48 h incubation and were confirmed by analyses performed at both earlier (24 h) and later (72 h) times after stimulation.
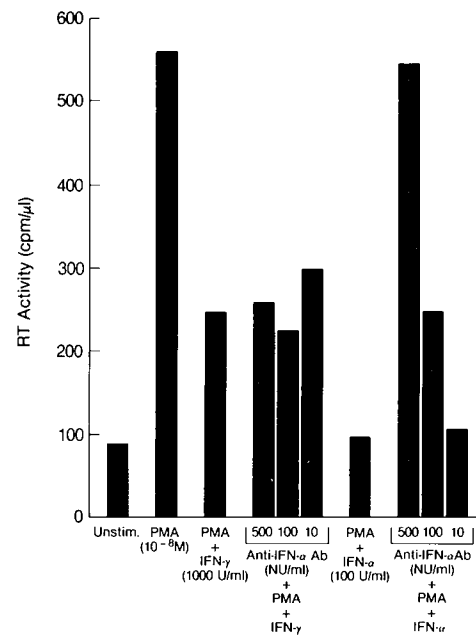

Figure 9. IFN-γ inhibition of PMA-induced HIV expression is not mediated by the release of endogenous IFN-α. U1 cells were stimulated with PMA in the presence or absence of IFN-α or IFN-γ, either alone or in combination with various concentrations of a sheep anti-IFN-α polyclonal Ab (Interferon Sciences, Inc., New Brunswick, NJ). The results refer to the levels of RT activity detected after 48 h of incubation, when the inductive effect of IFN-γ alone was not yet detectable. No effect of the anti-IFN-α Ab was seen on cells treated with PMA plus IFN-γ even at later time points.

with the accumulation of spliced and unspliced HIV mRNAs as well as with viral protein synthesis. In addition, IFN-γ induced the differentiation of U1 cells along the MP lineage in association with features of intracytoplasmic HIV accumulation in Golgi-derived vacuoles. Furthermore, IFN-γ greatly enhanced the intracellular accumulation of HIV concomitant with a significant reduction of plasma membrane-associated virions in U1 cells costimulated with PMA, a potent inducer of both HIV transcription and macrophage differentiation.

Several cytokines have been described as being capable of either upregulating (i.e., IL-2, IL-3, IL-6, M- and GM-CSF, and TNF-α/β), suppressing (i.e., IFN-α/β), or exerting multiple and opposite effects (TGF-β and IL-4) on HIV expression (reviewed in references 21 and 37). With regard to IFN-γ, Koyanagi et al. (20) observed that stimulation of MDM with this lymphokine before infection induced viral replication, whereas IFN-γ treatment of previously infected MDM reduced HIV expression; however, no explanation for this functional dichotomy was provided, and different results were reported by other investigators (19). In the persistently infected U1 cells employed in the present study, we have investigated by both functional and ultrastructural studies the patterns of virus production after stimulation with IFN-γ alone or after costimulation with IFN-γ and PMA. The direct inductive effect of IFN-γ on HIV expression was correlated with cell differentiation and the appearance of virions in intracellular vacuoles, although these features were clearly present only in a minority of the cell population. In PMA-stimulated cells, IFN-γ caused a significant inhibition of virion-associated RT activity released into the culture supernatant as compared with cells stimulated with PMA alone. However, this effect was not explained by either a reduced synthesis of viral proteins or RNA. When the functional and morphological effects of IFN-γ were compared with those of IFN-α, we observed that both IFNs modulated virus production in persistently infected cells stimulated with PMA by either inhibiting the ability of the virus to be released from the plasma membrane (as previously seen in IFN-α-treated T lymphocytic cells and here demonstrated in monocytic U1 cells) or by causing an accumulation of virions in intracellular vacuoles (as here shown in U1 cells treated with either IFN-α or IFN-γ). These results suggest that the capability of IFN-γ to shift the preferential morphological site of virus maturation from the plasma membrane to the intracellular compartment leads to an apparent decrease in particle release, which in reality is a diversion of

virion production resulting in a relatively stable intracellular viral reservoir. In this regard, we have previously described that long-term in vitro cultures of bone marrow-derived human macrophages infected in vitro with HIV-1 contained abundant levels of vacuole-associated particles after several weeks of culture (51). Furthermore, other investigators have observed virus accumulation in intracellular compartments as well as liberation of substantial RT activity after cell disruption after several days of culture of primary infected MDM (28-30, 32). Finally, vacuole-associated HIV has also been described in the brain macrophages of HIV-infected individuals (31). The potential role of IFN-γ as an in vivo mediator of intracellular accumulation of virus particles in monocytes and macrophages of HIV-infected individuals remains to be established. However, the observation of elevated levels of IFN-γ (or of its related marker neopterin) in the plasma and CSF (5-7) as well as in the lymph nodes of HIV-infected individuals (12), where the number of infected cells actively expressing HIV is much higher compared with the peripheral blood compartment (52-54), suggests that IFN-γ may play a role in the in vivo regulation of HIV expression.

Although IFN-γ and TNF-α have now been clearly demonstrated to induce HIV expression in a variety of model systems, including IL-2-stimulated PBMC (14), earlier studies had indicated the possibility that these two cytokines, either alone or in combination, could exert some suppressive effect on virus replication (15). In addition, IFN-γ restored certain defective functions in vitro, such as the natural killer cell activity in PBMC of HIV-infected individuals (10, 55). Based on these and other observations (15-19), IFN-γ has been employed as a therapeutic agent both in the treatment of HIV infection and of AIDS-associated Kaposi's sarcoma (reviewed in references 56 and 57) with conflicting results. In some studies reduction of plasma levels of p24 Ag, improvement of immune function, and clinical course have been described (58), whereas exacerbation and progression of disease have also been reported (59). Our in vitro findings suggest that the inductive effects of IFN-γ on HIV-infected MP are complex and that the apparent suppressive effects on virus production may in fact represent merely a diversion of the preferential subcellular site of virion production from the plasma membrane to intracytoplasmic vacuoles. In addition, we have clearly demonstrated that IFN-γ alone can upregulate HIV expression in persistently infected MP cells. Thus, caution should be used in the design of therapeutic strategies involving IFN-γ as potential antiviral agent.

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