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# Retinoic acid mimics transforming growth factor β in the regulation of human immunodeficiency virus expression in monocytic cells

**(macrophage/cytokines/U1 cells/U937 cells/phorbol 12-myristate 13-acetate)**

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**ABSTRACT** **Retinoic acid (RA) exerts potent suppressive and upregulatory effects on human immunodeficiency virus (HIV) expression in mononuclear phagocytes, strikingly similar to the effects of the cytokine transforming growth factor β (TGF-β). RA significantly inhibited phorbol ester-mediated, but not tumor necrosis factor α-mediated, induction of HIV transcription in the chronically infected promonocytic U1 cell line. RA and TGF-β also completely suppressed the induction of virus production in U1 cells by interleukin 6 alone or in combination with glucocorticoids, which predominantly upregulate virus expression at the posttranscriptional level. Despite the close parallel to TGF-β-induced effects, no evidence was obtained that RA mediated its effect by inducing secretion of active TGF-β1, -β2, or -β3. As with chronically infected U1 cells, similar inhibitory effects were also observed in primary monocyte-derived macrophages previously infected with HIV and then exposed to either RA or TGF-β. In contrast, stimulation of monocyte-derived macrophages or U937 cells (the parental cell line of U1) with either RA or TGF-β prior to *in vitro* infection resulted in the enhancement of virus production. Given the already successful use of retinoids in the treatment of several malignancies and the present demonstration of their capability of blocking the induction of HIV expression in infected mononuclear phagocytes, it would be of interest to pursue the potential role of this class of compounds in the development of strategies aimed at the pharmacologic regulation of HIV expression.**

Retinoids such as all-*trans*-retinoic acid (RA) are known to regulate the proliferative and differentiative capacities of several mammalian cell types (for review, see ref. 1). Of particular interest are their potent differentiative effects on leukemic cells *in vitro* and *in vivo* (2–4) and their beneficial effects in the treatment of patients with cutaneous malignancies and preneoplastic lesions (5, 6). The mechanism of action of retinoids is dependent upon their specific binding to one or more classes of intracytoplasmic molecules belonging to the superfamily of steroid receptors, followed by translocation of the retinoid–retinoid receptor complex to the cell nucleus where it binds to specific consensus sequences present in the promoter/enhancer region of target genes (7, 8). Of interest, the pleiotropic cytokine transforming growth factor β (TGF-β) has also been reported to exert similar effects on the proliferation and differentiation of several cell types, including fibroblasts, keratinocytes, and hematopoietic cells, in contrast to its initial characterization as a tumor promoting agent (9–16). Although TGF-β is usually secreted in a latent form, production of active TGF-β has also been reported in keratinocytes (17) and promyelocytic HL-60 cells (18) after treatment with retinoids, suggesting the possibility that some of the retinoid-mediated effects could be accounted for by the production of endogenous TGF-β.

Both RA and TGF-β have been investigated independently for their potential role in human immunodeficiency virus (HIV) infection. *In vitro*, upregulation of virus replication has been reported in both U937 and HL-60 myelomonocytic cell lines infected in the presence of RA (19–21), in contrast to suppressive effects observed on HIV infection of the MT-4 T-cell line (22). Both suppressive (23) and inductive (23, 24) effects on HIV expression have resulted from TGF-β stimulation of monocytic and T-lymphocytic (25) cells. Enhanced production of latent TGF-β1 has been documented in monocyte-derived macrophages (MDMs) infected with HIV *in vitro* (26), whereas *in vivo* expression of TGF-β has been documented in the brain tissues of HIV-infected individuals but not of HIV-negative subjects (26). Furthermore, increased secretion of active TGF-β1 by peripheral blood mononuclear cells isolated from HIV-infected individuals compared to healthy seronegative controls has also been reported (27, 28), suggesting that the increased production of this immunoregulatory cytokine may affect virus expression in infected individuals. Because of its multiple suppressive effects on the immune system (29–34), increased production of TGF-β may be an important determinant in several immune dysfunctions associated with HIV infection, including defective natural killer cell activity, monocyte–macrophage dysfunctions, and abnormal T- and B-lymphocyte responses (for review, see ref. 35).

In the present study, we have investigated the effects of RA on acute and chronic HIV infection of mononuclear phagocytes and compared them to those described for TGF-β. In addition, we have studied whether RA-mediated effects were accounted for by secretion of active TGF-β.

## MATERIALS AND METHODS

**Chronically Infected Cells.** The U1 promonocytic cell line has been described in detail. U1 cells were obtained by limiting dilution cloning of U937 cells surviving an acute infection with HIV-1 (LAV-1 strain) and are characterized by the presence of two copies of integrated HIV proviral DNA (36). Virus expression in U1 cells is constitutively low, although detectable levels of fully spliced and partially spliced viral mRNAs are present (37, 38). Incubation of U1 cells with phorbol 12-myristate 13-acetate (PMA) (Sigma) or cytokines such as tumor necrosis factor α/β (TNF-α/-β), granulocyte/macrophage colony-stimulating factor (GM-

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Abbreviations: RA, retinoic acid; TGF-β, transforming growth factor β; HIV, human immunodeficiency virus; TNF-α, tumor necrosis factor α; IL-6, interleukin 6; MDM, monocyte-derived macrophage; PMA, phorbol 12-myristate 13-acetate; GM-CSF, granulocyte/macrophage colony-stimulating factor; RT, reverse transcriptase; r, recombinant.

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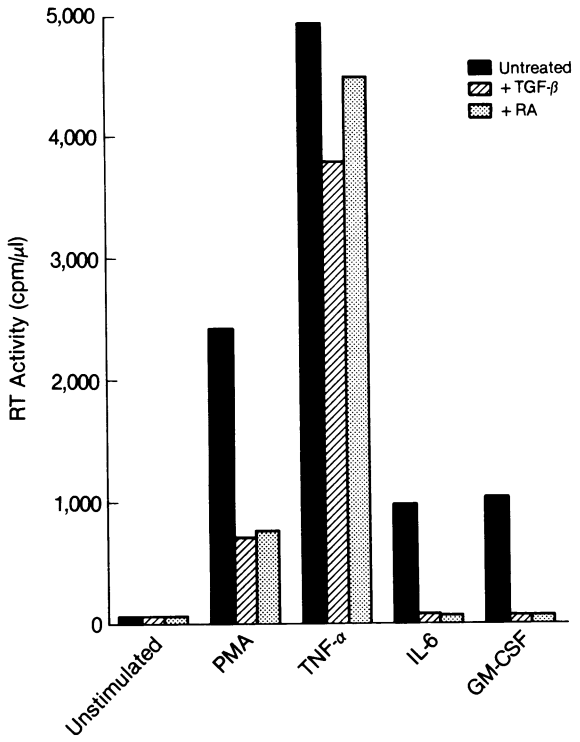


FIG. 1. Comparative suppressive effects of RA and TGF-β on the induction of HIV expression in U1 cells. Similar levels of suppression were observed throughout 7 days of culture in the presence of RA or purified native or recombinant TGF-β1, -β2, or -β3 and were confirmed by determinations of the levels of p24 antigen production, as measured by a commercially available ELISA kit (Coulter, Hialeah, FL). RA showed a concentration-dependent effect on virus production between 0.01 and 1 nM. Inhibition of HIV production in similar stimulatory conditions was also observed, although with lower efficacy, with other retinoids such as *N*-ethylretinamide (kindly provided by M. B. Sporn, National Cancer Institute, National Institutes of Health). The data shown are representative of five experiments. Variability of replicate cultures was always <15%.

CSF), and interleukin 6 (IL-6) results in the activation of HIV production (for review, see ref. 35). Typically, U1 cells ($2.5 \times 10^5$ cells per ml) were resuspended in RPMI 1640 medium (Whittaker Bioproducts) containing 10% (vol/vol) fetal calf serum (complete medium), plated in 96-well plates (Costar), incubated for 12 h with either RA (0.01–1 nM) or purified TGF-β1 (0.1 nM) (kindly provided by M. B. Sporn, NCI, NIH), and then stimulated with 0.1 μM PMA (Sigma), recombinant (r) TNF-α (100 units/ml) (Genzyme, Boston, MA), rIL-6 (100 units/ml) (Amgen Biologicals), or rGM-CSF (100 units/ml) (Genzyme), in the presence or absence of 10 nM dexamethasone (Sigma), at 37°C in 5% $CO_2$/95% air for 72 h.

**Acute HIV Infection of U937 Cells.** Mycoplasma-free U937 cells, obtained from the American Type Culture Collection, were incubated at $2 \times 10^5$ cells per ml in complete medium in the presence or absence of 1 nM RA or 1 nM TGF-β for 24 h before infection with HIV-1 (LAV-1 strain) and were maintained in medium alone or medium plus RA or TGF-β throughout the culture period.

**Infection of Primary MDMs.** Monocytes obtained from peripheral blood mononuclear cells of various seronegative donors were purified by overnight adherence to 24-well plastic tissue culture plates (Costar), resulting in $1 \times 10^6$ monocytes per ml per well, followed by elimination of the nonadherent fraction (containing predominantly T lymphocytes) by vigorous washing and pipetting. Monocytes were maintained 5–7 days before infection with a macrophage-tropic strain of HIV (ADA) (39) in Iscove's modified Dulbecco's medium (GIBCO), containing 10% fetal calf serum (basic medium), as described (23, 40). RA or TGF-β were added to part of the cultures before and throughout the entire culture period after infection (pretreatment protocol), whereas other cultures were incubated with the cytokines only 7–10 days after infection and throughout the subsequent culture period (posttreatment protocol). Approximately 50% of the culture supernatants was replaced with basic medium or medium containing TGF-β or RA every 5 days for several weeks of culture, and supernatants were collected and tested for the presence of reverse transcriptase (RT) activity. No gross differences in terms of cell number, adherent phenotype, and viability were observed in the various conditions used.

**RT Activity Assay.** Supernatants (5 μl) were added in duplicate to 25 μl of a mixture containing poly(A), oligo(dT) (Pharmacia), $MgCl_2$, and $^{32}P$-labeled dTTP (Amersham), and incubated for 2 h at 37°C. The mixture (6 μl) was spotted onto Whatmat DE81 paper, air-dried, washed five times in 2× standard saline citrate (SSC), and two additional times in 95% ethanol. The paper was then dried and cut, and radioactivity was measured with a Beckman LS 7000 scintillation counter.

**Northern Blot Analysis.** U1 cells were unstimulated, stimulated for 36 h with 1 nM RA, or preincubated for 12 h with

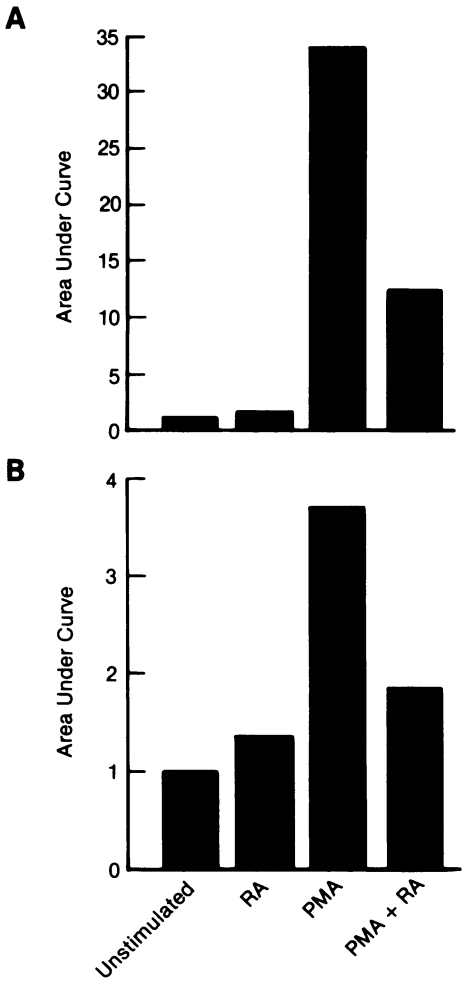


FIG. 2. RA suppresses HIV RNA synthesis and accumulation in U1 cells stimulated with PMA. (*A*) Northern blot analysis of steady-state HIV RNA accumulation. Laser-scanning densitometric analysis was performed using an LKB 2222-10 apparatus (LKB). The results are shown as ratios of the areas under the curve of the scanned HIV RNA bands normalized to those of the $\beta_2$-microglobulin controls, giving the arbitrary value of 1 to the ratio measured in unstimulated conditions, as described (23, 40). (*B*) Nuclear run-on analysis of HIV transcription. Densitometric laser scanning analysis of the levels of HIV RNA normalized to those of β-actin RNA was performed as described above.

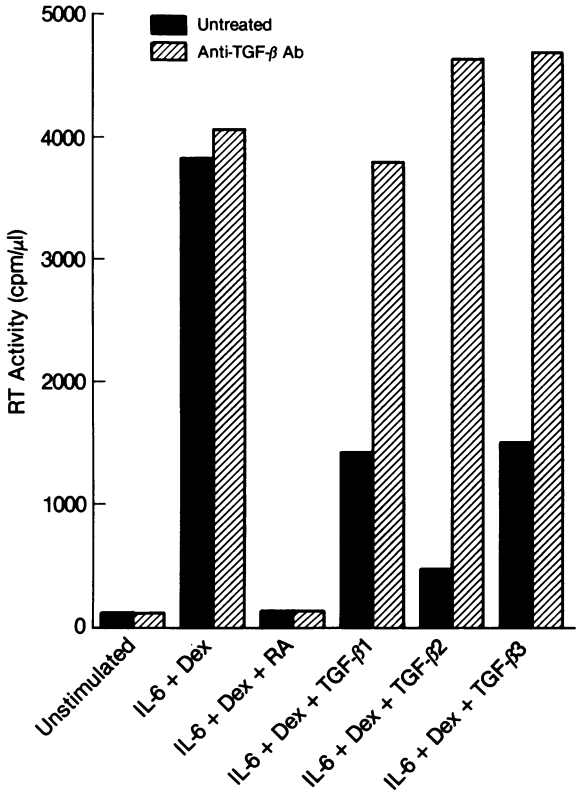


FIG. 3. RA suppression of HIV induction in U1 cells is not dependent upon secretion of active TGF-β1, -β2, or -β3. U1 cells were synergistically costimulated with IL-6 (100 units/ml) and dexamethasone (Dex; 10 nM) after a 12-h preincubation with 1 nM RA, 20 pM purified TGF-β1, 20 pM purified TGF-β2, or 1 pM rTGF-β3 (kind gift of M. B. Sporn), in the presence or absence of purified rabbit polyclonal antibodies (Ab) (20 μg/ml) raised against rTGF-β1 and -β2 (R & D Systems, Minneapolis, MN). Similar results were obtained with lower concentrations of antibodies (5 μg/ml) and when cells were incubated with higher concentrations (up to 1 nM) of TGF-β isoforms or lower concentrations of RA (0.1 nM).

RA and then stimulated for 24 h with 0.1 μM PMA. Total RNA was extracted from $1 \times 10^8$ U1 cells by the guanidine thiocyanate/phenol method with an RNA isolation kit (Stratagene). Total RNA (10 μg) was loaded per lane on a 0.8% agarose/formaldehyde gel, electrophoresed, and transferred to nitrocellulose by Northern blotting. The filters were baked and hybridized for 12 h with a $^{32}$P-labeled HIV long terminal repeat (*Hin*dIII–*Ava* I or *Sst* I–*Bss*HII) probe. 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/0.1% SDS; then, the filters were rehybridized with a $^{32}$P-labeled $\beta_2$-microglobulin cDNA probe.

**Nuclear Run-On Analysis.** Nuclei from $5 \times 10^7$ U1 cells were isolated after a 26-h incubation with 1 nM RA or a 14-h stimulation with PMA after a 12-h incubation with RA. Nuclear run-on was performed as described (41). Equal amounts of $^{32}$P-labeled RNA were hybridized to linearized PUC19 (control) or pNL4-3 [containing a full-length HIV genome (42)] plasmid DNA probes and to a human β-actin cDNA probe immobilized on nitrocellulose filters, which were then exposed to x-ray film.

## RESULTS AND DISCUSSION

No detectable virus expression was seen in chronically infected promonocytic U1 cells exposed to several concentrations (between 1 μM and 1 pM) of either RA or TGF-β. However, a significant inhibition (50–90%) of PMA-induced HIV production was observed in cells cotreated or pretreated (up to 24 h) with 0.1–1 nM RA or TGF-β (Fig. 1). In contrast, only a marginal reduction (0–20%) of virus production was observed when RA-treated U1 cells were stimulated with TNF-α, similar to what had been described with TGF-β (22) (Fig. 1). Complete suppression of HIV expression by either RA or TGF-β was also seen in U1 cells stimulated with IL-6 or GM-CSF (Fig. 1). These inhibitory effects of RA or TGF-β on virus production, as measured by particle-associated RT activity (43), were correlated with a partial (for PMA-stimulated cells) or complete (for IL-6- or GM-CSF-stimulated cells) suppression of HIV protein synthesis, as determined by Western blot or indirect immunofluorescence analyses (data not shown). Furthermore, RA treatment of PMA-stimulated, but not TNF-α stimulated (data not shown), U1 cells resulted in a significantly diminished accumulation of steady-state HIV mRNAs (Fig. 2*A*) and in reduced levels of viral transcription, as demonstrated by nuclear run-on analysis (Fig. 2*B*), as described in TGF-β-treated cells (23). No significantly enhanced transcription of viral RNA was observed in U1 cells stimulated with either IL-6 or GM-CSF even when cells were synergistically stimulated with glucocorticoids, although significant levels of HIV proteins and particles were produced [a phenomenon that we referred to as posttranscriptional induction (40)]. Therefore, no RNA studies were performed on RA- or TGF-β-treated cells under these stimulatory conditions.

Having established that the potent antiviral effects of RA in U1 cells closely resembled those of TGF-β, we investigated whether RA effects were mediated by the secretion of an active form of TGF-β, as described in other experimental systems (17, 18). No evidence of TGF-β activity was found in the culture supernatants of either unstimulated or RA-treated cells using a variety of stimulatory conditions, although U1 cells showed constitutive levels of TGF-β1 mRNA that were enhanced by PMA but not RA treatment (data not shown). Neutralization experiments were performed with both monoclonal and polyclonal antibodies directed against various isotypes of TGF-β known to be produced by human

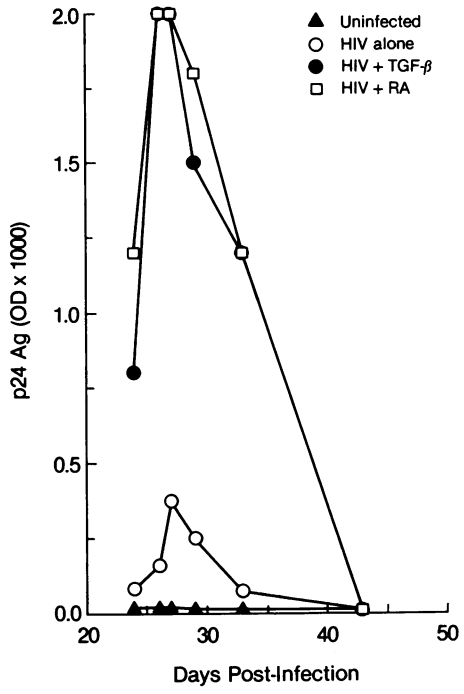


FIG. 4. RA or TGF-β upregulate HIV replication in acutely infected U937 cells. U937 cells were incubated with either 1 nM RA or 1 nM TGF-β1 24 h before infection with HIV-1. Similar results were obtained in three experiments and were confirmed by RT determinations. An $OD_{450}$ of 2 represents the upper limit of detection of the ELISA reader. Ag, antigen.

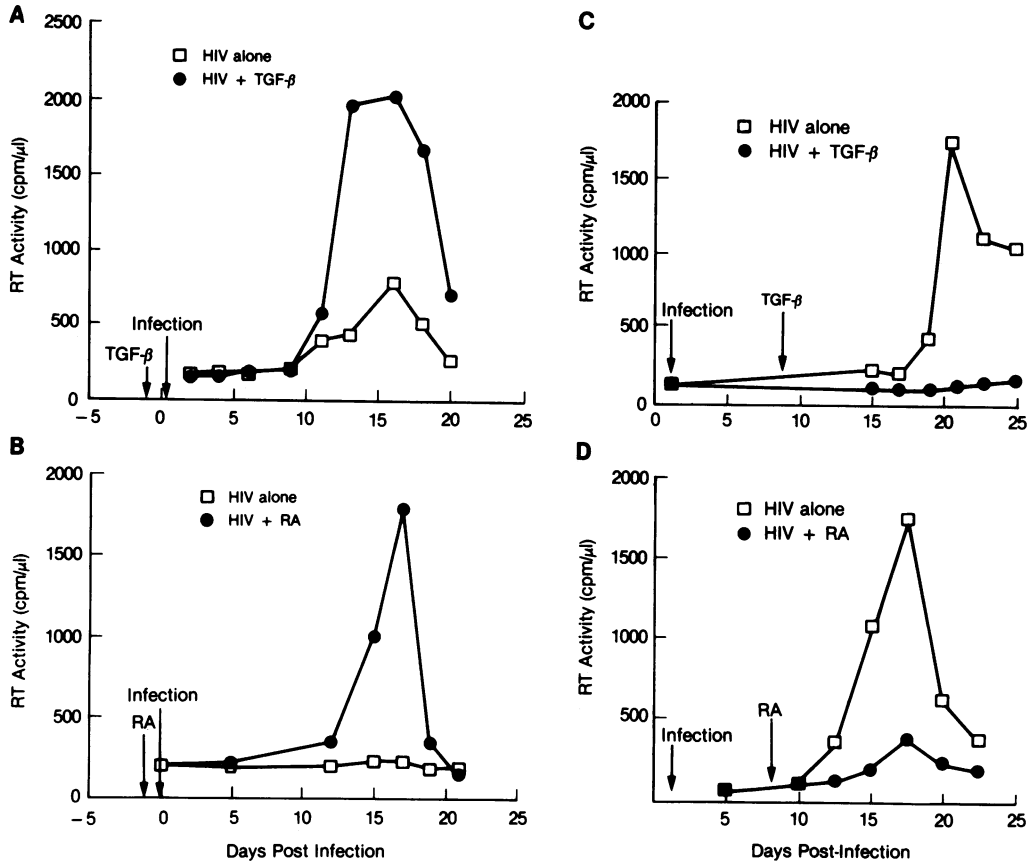


FIG. 5. RA or TGF-β exert dichotomous effects on HIV expression in primary MDMs infected *in vitro*. MDM cultures were incubated with 40 pM TGF-β1 (*A*) or 10 nM RA (*B*) 24 h before HIV infection and maintained with medium enriched with RA or TGF-β throughout the entire culture period. Independent cultures that were not treated with TGF-β or RA before or during infection were stimulated with these agents 7 days after infection (*C* and *D*, respectively) and were, further, maintained in the presence of the same concentrations of RA or TGF-β throughout the culture period.

cells. Polyclonal antibodies were very effective in neutralizing the inhibitory effects of exogenous TGF-β1, -β2, or -β3 on the induction of HIV expression in U1 cells; however, these antibodies did not reverse the suppression mediated by RA (Fig. 3). Thus, it is unlikely that RA-induced inhibition of HIV expression in U1 cells is dependent upon the secretion of active TGF-β1, TGF-β2, or TGF-β3.

We have reported (23) that TGF-β exerted a suppressive effect on HIV expression in primary human MDMs that had been infected *in vitro*, whereas it upregulated virus production if the cells were maintained in the presence of this cytokine several days prior to infection. Other investigators have confirmed both the suppressive effect of TGF-β on viral transcription (B. Ensoli, personal communication) and its inductive effect on virus replication in primary MDMs (24). It has also been reported independently that RA treatment of acutely infected myelo-monocytic cell lines such as U937 (the parental cell line of the persistently infected U1 cells) and HL-60 resulted in an inductive effect on virus production (19–21) [whereas virus production was suppressed in MT4 T cells (22)]. We confirmed these findings in that pretreatment of U937 cells with either RA or TGF-β resulted in a significant increased production of virus (Fig. 4). Furthermore, we have extended these observations to the primary MDM system, despite the fact that a significant variability exists in the susceptibility to HIV infection in cultures established from different seronegative donors (23, 40). Pretreatment with TGF-β or RA resulted in increased expression of HIV in cultures that showed poor ability to support HIV replication in the absence of these factors (Fig. 5 *A* and *B*), whereas treatment with RA or TGF-β several days after infection resulted in a significant down-modulation of virus production in MDM cultures that had been permissive of HIV replication (Fig. 5 *C* and *D*). These dichotomous effects of RA or TGF-β on either the U937/U1 cell lines or primary MDM cultures were not accounted for by gross changes in the proliferation, adherent phenotype, or viability, as discussed (23, 40), or by modulation of the CD4 molecule on the cell surface of target cells (data not shown).

Thus, RA resembles TGF-β functionally in its ability to regulate HIV expression in mononuclear phagocytes, as observed in non-HIV-related studies on the proliferative and differentiative effects of these two agents on both normal and neoplastic cells (10–13, 17, 18). The observation that RA or TGF-β treatment of mononuclear phagocytes resulted in opposite effects on virus production as a function of the experimental conditions reflects the biological complexity of these agents. Although TGF-β was originally described as a factor inducing anchorage-independent growth of several nonmalignant cells (14), it also counteracts certain tumor promoting agents and/or potentiates the antiproliferative effects of retinoids on epithelial tumor cells (10–13). With regard to HIV infection, our results and those obtained by others (19–25) suggest that the regulation of virus expression in mononuclear phagocytes may be dichotomous depending upon the phase of infection and/or the state of activation and differentiation of the cells at the time of infection. In this regard, opposite effects on virus production were seen in primary MDMs stimulated with interferon γ before or after HIV infection (44), whereas stimulation with interleukin 4 enhanced HIV replication in freshly isolated monocytes but suppressed HIV expression in 5-day-old MDMs (45).

In conclusion, the observation that RA exerts multiple and dichotomous regulatory effects on HIV expression in mononuclear phagocytes with a pattern closely resembling that of an important endogenous cytokine, TGF-β, which has been implicated at multiple levels in the pathogenesis of HIV infection *in vitro* (23–26) and *in vivo* (26–28), has several potential implications. Since the half-life of TGF-β *in vivo* is very short (46), long-term systemic administration of TGF-β is difficult at this time. However, the fact that retinoids are currently used for the therapy of several forms of human malignancies (2–6), including HIV-associated Kaposi sarcoma (47), coupled with the demonstration of important regulatory effects of these agents on HIV replication and expression, although complex and dichotomous, suggest that this class of compounds may have a potential role in therapeutic strategies employing pharmacologic modulation of HIV expression.

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