Effect of Retinoic Acid on HL-60 Cells Infected With Human Immunodeficiency Virus Type 1

By Marianne Semmel, Antonio Macho, Dominique Coulaud, Abdelkrim Allieche, Stéphane Plaisance, Jose Aguilar, and Claude Jasmin

HL-60 cells infected with human immunodeficiency virus type 1 (HIV 1) can be induced to differentiate along the granulocyte pathway by retinoic acid. In these cells, HIV mRNA synthesis is stimulated, but synthesis of viral proteins and virus replication are blocked and HIV-infected cells die after becoming apoptotic and/or vacuolized. © 1994 by The American Society of Hematology.

Cells of the monocyte/macrophage lineage are considered to be a reservoir of human immunodeficiency virus (HIV). The virus infects these cells without killing them and the cells produce moderate amounts of virus indefinitely. During the maturation of the cells, HIV replication can be modified. In particular, HL-60 cells induced to differentiate before infection become permissive for monocytotropic strains of HIV type 1 and infection with HIV can modify the capacity of the cells to differentiate in response to inducers. U937, a monocyte/macrophage cell line, has been studied extensively. Differentiation of these cells upregulates HIV expression at the level of HIV mRNA and virus production.

Modification of HIV expression can be mediated by several cytokines: tumor necrosis factor α (TNFα) upregulates HIV replication in a T-cell line and in U937 cells; the interferons (IFNs) downregulate it. However, IFNγ can upregulate HIV replication if added to the culture before infection. Transforming growth factor β (TGFβ) also has dichotomous effects. If added to differentiating monocytes, it upregulates HIV replication but downregulates it in immature monocytes and modifies HIV replication after induction of the cells with some but not all, inducers. Retinoic acid appears to act through TGFβ. Turpin et al. report an increase of HIV mRNA transcription in monocytes, the monocyte-like U937, and the myeloid THP-1 cells treated with retinoic acid. Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been shown to upregulate HIV expression in U1 cells (a clone derived from U937 cells), but to downregulate it in the parent line, U937. Interleukin-1 (IL-1), IL-3, and IL-6 can upregulate HIV expression. Upregulation of HIV expression appears to be linked to the activation of protein kinase C, but also to the induction of NFkB. Differentiation induces the production of cytokines in cells of the monocyte/macrophage lineage; their amount will depend on the response of the cells to the inducer and the infecting virus.

Because the relation between cytokines, differentiation, and HIV infection are very complex and because most of the studies have been performed with U937 cells that differentiate into macrophage-like cells, it seemed worthwhile to investigate the less intensively studied HL-60 cells, a line of myeloid parentage that can be induced to differentiate into granulocyte-like cells by retinoic acid but is less differentiated than THP1 cells. HL-60 cells are permissive for some but not all strains of HIV. We report here on the effect of retinoic acid on HL-60 cells infected with HIV1 and on virus replication in these cells.

Materials and Methods

Cells and virus. HL-60 cells were a gift from G. Milon (Institut Pasteur, Paris, France). CEM clone 11, a T-cell line, was a gift from F. Barré-Sinoussi (Institut Pasteur). HT4-LacZ1 cells were a gift from F.P. Garrick (Institut Pasteur). CEM and HL-60 cells were grown in RPMI 1640 medium (GIBCO, BRL, Gaithersburg, MD), containing 10% heat-inactivated fetal calf serum (GIBCO), 2 mmol/L L-glutamine, 100 U penicillin, 10 μg streptomycin, and 0.25 μg amphotericin B/mL. HT4-LacZ1 cells were grown as described by Rocancourt et al. According to the supplier, endotoxin levels are very low in the medium; the lot of serum used contained 43.8 ng endotoxin/mL. Cells were seeded at 2 × 10^5 cells/mL and were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Medium was renewed and cell concentration readjusted to 2 × 10^5 cells twice weekly. HIV type 1 strain LAI (formerly BRU) was a gift from F. Barré-Sinoussi. Stock virus was grown on CEM clone 11 cells and stored at −80°C. HL-60 cells were infected by adding cell-free supernatant (2 × 10^5 cells/mL) to the culture. Cell-free supernatant was stored at −80°C. To obtain chronically infected cells, HL 60 cells were passaged for at least 70 days after infection. HIV infection was assayed weekly by coculture with HT4-LacZ1 cells. Clones of chronically infected cells were obtained by limiting dilution and subcloned once. Continuing infection by HIV was monitored twice weekly by coculture with HT4-LacZ1 cells. To induce differentiation, cells were grown in the presence of 1 μmol/L all-trans retinoic acid (Sigma, St. Louis, MO). Cell viability was monitored by trypan blue exclusion.

For fluorescence-activated cell sortor (FACS) analysis, cells were washed with phosphate-buffered saline (PBS) fixed with 1% formaldehyde and 0.1% glutaraldehyde, colored with iodine, and analyzed with an EPICS Profile II Analyzer ( Coulter, Hialeah, FL).

To observe cell morphology, 2 × 10^5 cells were deposited on slides with a cytopsin centrifuge (Shandon; BioBlock, Paris, France), fixed with May-Grünwald reagent (Biolyon, Lyon, France), colored with the Giemsa reagent (Prosciences, Paris, France), and visualized in a Leitz Diaplan 20 microscope using oil immersion.

For electron microscopy, cells were fixed in 4% glutaraldehyde in PBS and pelleted at low speed. The pellet was washed in 67 mmol/L phosphate buffer, pH 7.4, postfixed in 2% osmium tetroxide,
dehydrated with ethanol, and included in Epon resin by the usual techniques. Sections of cells were colored with uranyl acetate and lead citrate and observed with a Zeiss EM 902 microscope. Enhanced contrast was obtained by selecting elastic electrons using the slit of the spectrophotometer.

**Determination of reverse transcriptase and tissue culture infectious units.** HIV replication was assayed by measuring reverse transcriptase activity in the supernatant according to Oysten-Jonassen.21 Briefly, 50 µL of cell-free supernatant was incubated for 12 hours at 37°C in a mixture containing 0.05% Triton X100, 50 mmol/L KCl, 3 mmol/L dithiothreitol, 30 mmol/L Tris, pH 7.8, 0.3 mmol/L EGTA, 0.13 U poly-rA-dT as primer (Pharmacia, Brussels, Belgium), and 0.0378 nCi 3H-deoxythymidin triphosphate (Amersham, UK). The reaction was stopped with 10% trichloracetic acid containing 2 mmol/L Na pyrophosphate. The precipitate was washed 6 times with 5% trichloracetic acid containing 12 mmol/L Na pyrophosphate, using an Automash 2000 Harvester (Dynatech; BioBlock) and Skatron filters. The filters were counted in scintillation liquid in a Beckman counter. Tissue culture infectious units (TCIU) were determined by titrating the virus in the supernatant or in the cells according to Rocancourt et al.29 Briefly, cell-free supernatants or cell suspensions were added to cultures of HT4-Lac Z1 cells (transfected with HIV 1 LTR linked to β-galactosidase) and incubated for 3 days; the cells were then fixed with formaldehyde and glutaraldehyde and incubated with the substrate of β-galactosidase. Multiplying HIV produces tat, which activates HIV LTR and the β-galactosidase revealed with the substrate. The resulting blue syncytia correspond to the number of replication-competent virus in the supernatant.

**Nucleic acid and protein assays.** DNA was extracted according to Sambrook et al.32 from 10⁶ cells. HindIII fragments of λ phage DNA were used as size markers. RNA was extracted from 2 to 3 × 10⁷ cells and Northern blotting was performed according to Chomczynski and Sacchi,33 using a PHTI probe corresponding to the entire genome of HIV (a gift from M.C. Lang, Institut Pasteur). To analyze proteins, a pellet of 2 × 10⁶ washed cells was suspended in 50 µL Laemmli's buffer34 and heated for 3 minutes at 100°C. Sodium dodecyl sulfate polyacrylamide gels 10% to 15% were run using the Phast system (LKB-Pharmacia) and blotted on nitrocellulose membranes (Schleicher & Schull, Keene, NH) using the Phast transfer unit. Membranes were saturated with 5% bovine serum albumine fraction V (Boehringer, Mannheim, Germany) in PBS, incubated overnight with the primary antibody, washed and incubated with biotinylated antispecies antibody, washed and incubated with streptavidin linked to alkaline phosphatase, and shown with the appropriate substrate according to the instructions from the supplier (Amersham). Rainbow molecular weight markers were purchased from Amersham. HIV anti-gag and anti-env (anti-gp41 and anti-gp120) antibodies were purchased from TEBU (Le Paray en Yvelines, France).

**RESULTS**

Figure 1A shows the effect of retinoic acid on growth and viability of HIV-infected and uninfected HL-60 cells. Growth of uninfected cells is slowed down by retinoic acid, but is resumed slowly after the inducer is removed (Fig 1A). Viability is higher than 80% at all times (Fig 1B). Infected cells grow at the same rate as uninfected cells (Fig 1A). When infected cells are grown with retinoic acid, they cease to multiply after 4 days and die 14 to 18 days later (Fig 1A). Viability decreases 11 to 14 days postinduction (Fig 1B). When cells are chronically infected and treated with retinoic acid the same effects are observed (data not shown). Growth and viability of infected cells decreases transiently 11 to 14

| Table 1. FACS Analysis of HL-60 Cells Treated With Retinoic Acid |
|---------------------------------|-------|-------|-------|-------|
| Mean diameter (µ) of "large" cells | HL-60 | HL-60 + RA | HL-60 HIV | HL-60 HIV + RA |
| Mean diameter (µ) of "small" cells |       |       |       |       |
| % "Large" cells | 25.6  | 23.6  | 30.6  | 24.8  |
| % "Small" cells |       |       |       |       |
| Total | 91.4  | 88.4  | 92.8  | 59.4  |
| Excluding iodine | 8.2   | 31.6  | 7.1   | 40.6  |
| Excluding iodine | 7.5   | 4.5   | 0.4   | 7.4   |

Cells were harvested 8 days postinduction; retinoic acid was present for 7 days. Five thousand cells per sample were tested. Abbreviation: RA, retinoic acid.
Fig 2. Electron micrograph of HL-60 cells at low magnification. Cells were harvested 8 days postinduction. Scale bars = 10 μm. (a and b) Untreated; (c and d) treated with retinoic acid. Uninfected (a) and infected (b) cells are undistinguishable; after treatment, most uninfected cells (c) have normal density, nuclei are multilobular, and two cells are apoptotic with dense cytoplasm, surface blebbing, and electron dense micronuclei. All infected cells (d) are apoptotic with dense vacuolized cytoplasm; apoptotic bodies are frequent.
Fig 3. Electron micrograph of HL-60 cells at high magnification. Cells were harvested 8 days postinduction. Scale bars for (a, b, d, and e) are 1 μ; for (c and f) are 0.1 μ. (a, c, and d) Untreated; (b, e, and f) treated with retinoic acid. (a) Uninfected cell; (b) uninfected cell, note multilobular nucleus and beginning chromatin condensation along the nuclear membrane; (c) cell from virus producing clone 8, with budding virus (arrows); (d) detail of mature and immature virus; (e) HIV-infected cells, virus-producing clone 8, note the group of virions that may have been produced by the apparently normal cell but could also have been released from a lysed cell because they are associated with cellular debris; (f) detail of budding and mature virus without double membrane and knobs.
Fig 4. Special features of HIV-infected cells (non-virus-producing clone 9) treated with retinoic acid. Scale bars = 1 μ; inset of (g) scale bar = 0.1 μ. (a and b) Apoptotic cells. (b, c, and e) Detail of (a) and (d); micronuclei with filamentous structures and condensed chromatin; note that the nuclear outer membranes are detached. (f, g, and h) Filamentous structures found in micronuclei (f and g) and in cytoplasm (h); the inset in (g) shows a transverse cut of filaments (diameter, 5 nm).
Table 2. Effect of Retinoic Acid on HL-60 Cell Morphology

<table>
<thead>
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<th>Cells</th>
<th>Before Induction</th>
<th>8 Days Postinduction</th>
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<tr>
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<td>22</td>
</tr>
<tr>
<td>HL-60, HIV-infected clone 8, producing virus</td>
<td>31</td>
<td>72</td>
</tr>
<tr>
<td>HL-60, HIV-infected, clone 9, producing few virions</td>
<td>5</td>
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One hundred cells were counted.

days postinfection (Fig 1A and B and Semmel et al32). HL-60 cells treated with retinoic acid, whether infected or not, assume a granulocyte like morphology. Up to 80% of the cells appear to be differentiated between days 4 and 11 postinduction. Only 6% of the uninfected cells are differentiated 21 days postinduction; the percentage of differentiated infected cells also decreases, but these cells die 14 to 18 days postinfection. On day 7 postinduction, many of the infected cells that do not exclude trypan blue appear to be smaller than the corresponding uninfected cells. We therefore analyzed chronically infected and uninfected cells 8 days postinduction by FACS. HL-60 cells, whether infected or not, are heterogeneous in respect to size and constitute two subpopulations. HIV-infected cells are larger than uninfected cells; treatment with retinoic acid causes a decrease of the size, and the percentage of smaller cells increases. Iodine penetrates cells more readily after treatment and when the cells are “large.” The results of these experiments are summarized in Table 1.

Chronically infected cells were cloned. All clones had subpopulations of “large” and “small” cells, but differed in respect to virus production as determined by coculture with HT4-LacZ1 cells. Some clones consistently produce large amounts of virions, others produce, occasionally, a few virions. Electron microscopy (Figs 2 through 4) shows that treatment with retinoic acid decreases the size of uninfected HL-60 cells. The nuclei become horseshoe shaped and crenellated, resembling the nuclei of granulocytes. In some cells, chromatin is segregated, a few cells are vacuolated, and there are some apoptotic bodies (Fig 2c). At low magnification, infected cells are indistinguishable from uninfected cells before treatment with retinoic acid. At high magnifications, budding virions can be seen at the membrane of infected cells (Fig 3c) and most of the extracellular virions have an electron-dense double membrane and knobs (Fig 3d). After retinoic acid treatment, the cells and many cells are vacuolated (Fig 2d), whether or not the cells produce virus. Budding virions are rare and there are no extracellular virions with double membranes (Fig 3e and f). The nuclear membrane is often detached (Fig 4e) and, in addition, both cytoplasm and nuclei contain filaments (Fig 4). To our knowledge, such formations have not been described previously. Their origin is unknown, but their presence appears to be related to apoptosis, because they have also been observed in apoptotic cells of other lymphocytic cell lines (Ramos et al, personal communication, 1993). Table 2 summarizes quantitative results of electron microscopy.

Chromatin segregation and detachment of the nuclear membrane are considered to be a symptom of apoptosis, as is fragmentation of DNA in a typical “DNA ladder” pattern.35 DNA extracted from HL-60 cells chronically infected with HIV does not differ from DNA of uninfected cells. After induction with retinoic acid, less DNA per cell is extracted, but the migration pattern is the same whether the cells were infected or not, showing only a band of high molecular weight material (data not shown).

HIV replication is blocked in HL-60 cells grown with retinoic acid regardless of whether the cells were chronically

Fig 5. HIV replication in HL-60 cells. The experiments were repeated eight times and the results were consistent within each experiment. (A and C) Reverse transcriptase activity. (B and D) TCIU. (●) Uninduced controls; (●) induced with 1 μmol/L retinoic acid. Inducer was present for 7 days. (A and B) Cells were simultaneously infected and induced. (C and D) Chronically infected cells were induced.
A  
B  
C  

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*HIV signal too low for measurement
+Underestimated, HIV signal too strong for measurement

Fig 6. Dot blots of RNA extracted from HL-60 cells 9 days after induction. Inducer was present for 7 days. (A) Shown with a PBT probe. (B) The same blot shown with an actin probe. (C) Ratio of OD of dot hybridized with the PBT probe/OD of the same dot hybridized with the actin probe. Measured with a personal densitometer (Molecular Dynamics, Sunnyvale, CA). Uninfected cells; (b) cells infected with HIV on day 0; (c) cells simultaneously infected and induced with 1 pmol/L retinoic acid; (d) chronically infected cells; (e) chronically infected cells induced with 1 µmol/L retinoic acid.

RNA and proteins were analyzed when more than 75% of the cells were viable. HIV mRNA transcription increases in cells grown with retinoic acid (Fig 6, lanes c and e). The effect is more pronounced in chronically than in freshly infected cells (Fig 6c). This RNA appears not to be translated; in all samples less HIV-specific proteins are found in treated than in untreated cells (Fig 7, lane c). The env-coded proteins (Fig 7B and C) appear to decrease more than the gag-coded proteins (Fig 7A).

To exclude an unspecific effect of the inducer, we tested CEM cells, a T-cell line that produces HIV but that is not inducible. We found that retinoic acid does not modify HIV production or in vitro reverse transcriptase activity in these cells (data not shown).

DISCUSSION

Uninfected HL-60 cells multiply more slowly when grown with retinoic acid, but remain viable and resume growth after the removal of the inducer whereas HIV infected cells die 1 week after the removal of the inducer. In these cells, chromatin is segregated and the nuclear membrane is detached, suggesting that the cells are apoptotic, although the DNA is not fragmented. Apoptosis without activation of the endonuclease responsible for DNA fragmentation has been described in several cell lines. It is possible that HL-60 cells resemble these cells but it is also possible that the DNA extracted from induced cells represents only the DNA from normal or lyzed, but not from apoptotic, cells.

Retinoic acid upregulates HIV mRNA transcription in HL-60 cells as it does in monocytes and THP-1 cells. We do not find a corresponding increase of HIV replication; on the contrary, HIV replication is blocked. The decrease of HIV mRNA translation and virus replication could be the consequence of cell death, but the abnormal virions observed in cells grown with retinoic acid, the selective decrease of env-coded viral protein synthesis, and the difference between the loss of reverse transcriptase activity and the loss of replication competent virus suggest interference with HIV mRNA translation. At first view, our results and the results reported by Kitano et al appear to be contradictory. In the same system, ie, HL-60 cells infected with HIV 1 LAI, they observed an increase of HIV replication and some toxicity in infected (Fig 5C and D) or infected and induced simultaneously (Fig 5A and B). Retinoic acid added 4 days before infection has the same effect (data not shown).

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Fig 7. Western blots of proteins from HL-60 cells simultaneously infected and induced for 7 days, 10 days postinfection. (A), with anti-gag serum; (B), with anti-env (gp120) serum; (C), with anti-env (gp41) serum. (a) Uninfected cells; (b) infected cells; (c) infected cells induced with 1 µmol/L retinoic acid.
cells treated with retinoic acid. However, their conditions differed from ours; they treated the cells before infection, we treated them during and after infection. Also, the HL-60 strains used appear to be different. Only a subpopulation of the HL-60 cells used by Kitano et al expressed the CD4 antigen and not all cells were infected, whereas 92% of our HL-60 cells expressed this antigen and more than 95% of the cells were infected; therefore, one would expect more toxicity when more cells were infected.

The morphology of the cells shows that retinoic acid induces differentiation in both uninfected and HIV-infected cells and that undifferentiated cells overgrow the differentiated uninfected cells, whereas the HIV-infected cells die when the inducer is removed.

Many published results, including ours, indicate the existence, in cells of the monocye/macrophage lineage, of a stage of differentiation that restricts HIV replication at one or more levels of the replication cycle, whereas, at other stages of differentiation, HIV replication is upregulated. Each stage of differentiation corresponds to a balance of cytokines and it appears reasonable to expect that the outcome of HIV infection in a given cell (replication of the virus, silent infection, or cell death) will be determined by this balance. Retinoic acid, while stimulating HIV mRNA transcription, appears to block HIV replication and to cause the death of infected but not of uninfected HL-60 cells, possibly by modifying the balance of cytokines. Immature granulocytes are the normal counterpart of HL-60 cells. If the effect of retinoic acid on the normal cells in vivo were the same as that on HL-60 cells in vitro, retinoic acid would promote the death of HIV-infected cells, thereby reducing the reservoir of HIV-infected cells, and would cause the emergence of replication defective virus, thus reducing the virus load; it might then be considered useful for the treatment of acquired immunodeficiency syndrome. On the other hand, the absence of mature granulocytes might facilitate opportunistic infections because this cell is active in the host defense.

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