Journal of Cellular Biochemistry 45:366–373 (1991)

ARTICLES

Extrachromosomal Human Immunodeficiency Virus Type-1 DNA Can Initiate a Spreading Infection of HL-60 Cells

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Abstract In this report, we describe a human immunodeficiency virus type-1 (HIV-1)-infected promyelocytic cell line, OM, derived from HL-60 cells. Although the OM cell line was biologically cloned twice, the pattern of HIV-1 expression during culture appeared analogous to a classical acute spreading infection and was inhibited by both azidothymidine and recombinant soluble CD4 treatment. The number of OM cells actually expressing HIV-1 at the beginning of culture was 0%, reached a peak of nearly 100% at 6 weeks, and then fell to $<10\%$ HIV-1^+ cells by 10 weeks. Clonal analysis of the surviving cells verified that stable HIV-1^+ OM cells resulted from the spreading infection. Southern analysis confirmed the transmission of HIV-1 through these OM cultures and the occurrence of stable clones which resulted. The initial percentage of OM cells actually harboring the HIV-1 genome was $<0.1\%$, indicating nonfaithful transmission of an unintegrated HIV-1 genome during clonal expansion. These results demonstrate that extrachromosomal HIV-1 DNA can contribute to the spread of HIV-1 infection and give rise to cells which have stably integrated HIV-1 provirus.

Key words: HIV-1 infection of promyelocytes, unintegrated HIV-1 DNA, azidothymidine, CD4, clonal analysis

Establishment of an integrated proviral form is classically considered an initial step in the life cycle of the human immunodeficiency virus type-1 (HIV-1), as well as other retroviruses [1,2]. Once integrated, the proviral DNA remains dormant within the host genome, being maintained by host replication and transmission to daughter cells during cellular division. At some point, viral activation occurs resulting in the production and release of infectious virions to complete the life cycle [2].

However, additional nonclassical means of HIV-1 propagation have recently been described. An extrachromosomal HIV-1 genome, established by mutagenesis of the integrase gene, was transiently maintained once transfected into susceptible host cells. These unintegrated HIV-1 DNA forms were still capable of transcribing viral proteins but produced noninfectious virions [3]. Furthermore, unintegrated HIV-1 DNA has been observed due to a continual process of superinfection in both acute [4,5] and chronic [5,6] infections. The unintegrated DNA may contribute to the cytopathic effect of the virus [5]. In persistently infected promonocytic cells, extrachromosomal HIV-1 DNA forms were structurally stable and produced limited amounts of viral RNA [6]. However, it remains uncertain what contribution extrachromosomal DNA forms may make to the maintenance of HIV-1 during dormancy and the acute spread of infec-

Abbreviations used: HIV-1, human immunodeficiency virus type-1; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; IFA, immunofluorescence assay; RT, reverse transcriptase; AZT, azidothymidine; kb, kilobase.

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Received October 3, 1990; accepted December 14, 1990.

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tious virions to susceptible cells upon viral activation.

Through a series of subclonings, we were able to isolate an HL-60 promyelocytic clone harboring an extrachromosomal HIV-1 genome in an extremely low frequency of cells. This genome apparently was not integrated at the time of subcloning and, therefore, was not faithfully transmitted during clonal expansion. However, the virions produced from this extrachromosomal HIV-1 genome were still capable of causing an acute spreading infection of susceptible HL-60 cells. Our results confirm that extrachromosomal maintenance of HIV-1 is an important aspect of the virus life cycle and can contribute to the cellular pathophysiology of acquired immunodeficiency syndrome (AIDS).

MATERIALS AND METHODS

Cells and Culture Conditions

HL-60 [7] and A3.01 cells [8] were obtained from American Type Culture Collection (Rockville, MD). Both cell types were maintained in RPMI 1640 basal medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum, 2 mM glutamine, and 1% Pen-Strep (Gibco) at 37°C in a humidified atmosphere of 5% CO_2/95% air. All cell cultures were maintained in log-phase growth by diluting them 1:10 with fresh medium every 48–72 h. In some experiments, azidothymidine (AZT, obtained from Burroughs Wellcome through Dr. Charles R. Horsburgh, CDC) or recombinant soluble CD4 (obtained from Genentech through Dr. Steven McDougal, CDC) were added to cell cultures, at 10 μg/ml and 20 μg/ml, respectively.

HIV-1 Infection of HL-60 Cells

HIV-1 infection of HL-60 cells was achieved by a coculture procedure. A3.01 cells were first infected with HIV-1 (LAV) stock virus and cultured for 3 days, at which time cell aggregation was observed. These HIV-1-infected A3.01 cells were γ-irradiated (1,800 rads) and then cocultured at a 1:5 ratio with 1×10^7 log-phase HL-60 cells in 15 ml medium. The coculture was replenished with fresh medium every 3 days; on day 12 the surviving cells were cloned directly into U-bottom 96-well plates (Corning Glass Works, Corning, NY) by limiting dilution. The resultant colonies were tested for HIV-1 expression and further subcloned by limiting dilution.

Detection of HIV-1 Expression

Cell culture supernatants were examined by reverse transcriptase (RT) assay [9] and HIV-1 p24 antigen-specific enzyme-linked immunosorbent assay (ELISA) (Maryland Medical Laboratories, Baltimore, MD) for evidence of HIV-1 expression.

A direct immunofluorescence assay (IFA) for HIV-1 expression was performed on cells attached to AdhesioSlides (MM Developments, Ottawa, Canada). Attached cells were first fixed in 4°C acetone for 10 min and then rinsed with phospate-buffered saline (PBS). Cell fields were blocked with 5% normal goat serum for 45 min at room temperature, rinsed with PBS, and then reacted with a fluorescein-conjugated anti-HIV-1 polyclonal antiserum (provided by Dr. Steven McDougal, CDC) derived from AIDS patients. After an additional 45 min at room temperature, the slides were rinsed with PBS and cells were examined by ultraviolet light microscopy.

Determination of Cell Surface Phenotype

Cell surface phenotype determination was performed by flow cytometry as described [10], except that cytometric analysis was done on a Becton Dickinson FACScan system. Directly conjugated anti-CD13 (Coulter, Hialeah, FL) and anti-CD5 (Becton Dickinson, Mountain View, CA) monoclonal antibodies were used following the manufacturers' recommendations. Anti-CD4 (Ortho, Raritan, NJ) and anti-CD8 (Ortho) antibodies were used in an indirect manner with a phycoerythrin-conjugated goat-anti-mouse immunoglobulin secondary antibody (Tago, Burlingame, CA).

Polymerase Chain Reaction (PCR) for Cellular Frequency of HIV-1 Provirus

Serial log dilutions of OM or 8E5 [11] cells into uninfected HL60 cells were prepared so that each contained 1.5×10^5 total cells. Each dilution was lysed in 25 μl buffer (50 mM KCl, 10 mM Tris hydrochloride (pH 8.3), 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% Nonidet P-40, and 0.45% Tween-20). After lysis, PCR was performed using 100 ng of HIV-1-specific *gag* primers SK 38/39 [12] in 35 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min, as described [13]. Amplified products were analyzed by agarose gel electrophoresis and Southern hybridization to ^{32}P-end labeled SK19 probes [12].

Southern Blot Analysis

Total cellular DNA was isolated [14] from the individual cell types and digested with Eco-RI and Bam-HI restriction endonucleases. Digestion products were separated by agarose gel electrophoresis and analyzed by Southern hybridization to a ^{32}P-end labeled HIV-1 Eco-RI/Hind-III 2.4 kilobase (kb) fragment from p-BENN 6 [8].

RESULTS

Establishment of an HIV-1 Infected HL-60 Cell Clone

HL-60 cells were cocultured with γ-irradiated, HIV-1-infected A3.01 cells ($CD5^+$) to improve the efficiency of infectivity through cell-to-cell contact [15]. Surviving cells from this coculture were subcloned and a single RT^+ clone (cloning frequency = 17.7%) was identified. This RT^+ clone was immediately subcloned (cloning frequency = 36.5%) and >90% of the resultant populations were $HIV\text{-}1^+$ upon the initial p24 ELISA screening. However, the majority (28/35) of these subclones quickly lost constitutive HIV-1 expression after expansion into T25 tissue culture flasks. From the seven remaining $HIV\text{-}1^+$ subclones, one that secreted low level p24 antigen was chosen for further characterization. This subclone, designated OM, was determined by flow cytometry to be $CD13^+$ and $CD5^-$ (data not shown), verifying that it was not an HIV-1-infected A3.01 cell which had survived γ-irradiation [8].

As with the majority of other subclones, OM cultures lost constitutive HIV-1 expression during expansion for cryopreservation and became entirely $HIV\text{-}1^-$, by supernatant RT/p24 antigen levels (Fig. 1A) and IFA (Fig. 1B). Neither HIV-1-specific proteins, by immunoblot assay, nor HIV-1 mRNA, by Northern analysis, were resolvable from OM cell lysates (data not shown). However, in dealing with either the original or cryorecovered OM cultures, a predictable and reproducible pattern of increasing viral expression eventually developed (Fig. 1A).

Spontaneous HIV-1 Expression in OM Cultures

From the initiation of OM cultures, a 2- to 3-week period of viral dormancy existed. During this time, a small percentage of the clonal OM population spontaneously began to express HIV-1 (Fig. 1B). Over several additional weeks, OM cultures progressively converted toward increasing HIV-1 expression and reached a peak where >95% of the cells had become $HIV\text{-}1^+$ by IFA (Fig. 1B). This conversion was also evidenced by increasing supernatant RT/p24 antigen levels (Fig. 1A) and detection of both HIV-1-specific proteins and mRNA from cell lysates (data not shown). As OM cultures were maintained beyond this peak, the percentage of $HIV\text{-}1^+$ cells spontaneously declined (Fig. 1B), as did culture supernatant RT/p24 antigen levels (Fig. 1A), without noticeable cytopathicity. The loss of HIV-1 expression in OM cultures continued for several weeks until <10% of the cells remained stably identifiable as $HIV\text{-}1^+$.

For ease of reference, a numerical suffix (from 1 to 10) was assigned to OM cultures which indicated their progressive level of HIV-1 production (Fig. 1A). For instance, $HIV\text{-}1^-$ cryorecovered OM cultures were designated OM-2. As an OM-2 culture began spontaneous HIV-1 expression, the culture was then designated OM-4. OM-7 designated that this same culture had reached its peak of HIV-1 expression. Finally, an OM-10 designation indicated that the culture again contained less than 25% $HIV\text{-}1^+$ cells.

Acute Infection Accounts for the Spread of HIV-1 Through OM Cultures

Although the OM cell line had been biologically cloned twice, the progression of HIV-1 expression appeared similar to an acute infection of susceptible cells rather than a clonal activation of latent HIV-1 provirus. To determine if the spreading HIV-1 expression by progressive OM cultures had additional characteristics of a classical acute infection, CD4 surface expression was evaluated by flow cytometry (Fig. 2). OM-2 cells expressed CD4 at the same surface density as uninfected parental HL-60 cells. However, concomitant with spontaneous HIV-1 expression, a subpopulation of $CD4^-$ cells emerged in OM-4 cultures and progressively increased so that >95% OM-7 cells were $CD4^-$. OM-10 cultures became again predominantly $CD4^+$ (>80%) in accordance with the decline in HIV-1 expression, although a $CD4^-$ subpopulation persisted. Therefore, the pattern of CD4 surface downmodulation by progressing OM cultures also indicated a spreading HIV-1 infection [16].

Inhibition experiments verified the acute transmission of HIV-1 through the clonal OM population. OM-2 cultures maintained in the

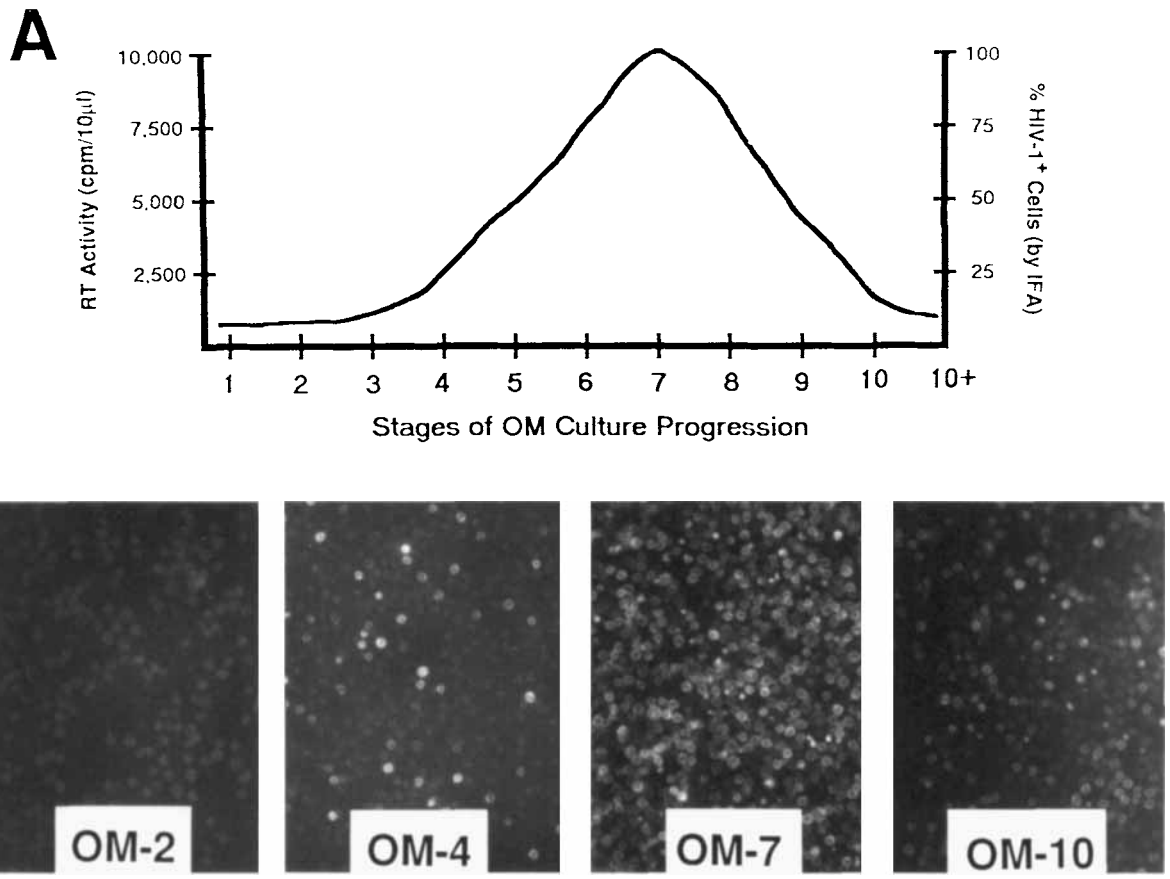

Fig. 1. Spontaneous HIV-1 expression by OM cultures. **A** represents the pattern of HIV-1 expression by progressing OM cultures. Data are presented for the increasing RT activity and percentage HIV-1$^+$ cells for an individual OM culture but are representative of more than 20 independent cultures. OM cultures are assigned a numerical suffix (from 1 to 10) to designate their level of progressive HIV-1 expression, as described in the text. This relative stage designation and not an absolute time scale is depicted along the abscissa. **B** demonstrates the typical IFA results for HIV-1 expression by several stages of an OM culture. HL-60 cells showed no reactivity with the anti-HIV-1 antiserum and the background fluorescence was identical to that of OM-2 cells.

presence of recombinant soluble CD4 were prevented from spontaneous HIV-1 expression, as determined by IFA, whereas untreated OM-2 cultures progressed characteristically to a peak of HIV-1 expression (data not shown). Similar results were obtained when OM-2 cultures were maintained in the presence of azidothymidine (AZT). RT activity of the culture supernatants confirmed these observations (Fig. 3); OM-2 cultures maintained with recombinant soluble CD4 or AZT continued to display background RT activity, whereas RT activity of control cultures rose predictably.

To determine if stable HIV-1 integration could result from the spreading acute infection, additional subclonings of OM-10 cultures were performed. As predicted by the low percentage of HIV-1$^+$ cells in the OM-10 cultures, upon subcloning very few of the expanded populations (3/45 = 6%) displayed HIV-1 expression by p24 antigen ELISA. None of the HIV-1$^-$ subclones tested (N = 4) were found to be harboring latent provirus when examined by PCR for HIV-1 *gag* sequences (data not shown). The HIV-1$^+$ subclone which produced the highest level of p24 antigen, designated OM-10.1, was further tested for evidence of genomic integration.

Southern Analysis for HIV-1 Genome in OM Cultures

Southern analysis was performed to confirm the transmission of HIV-1 through OM cultures (Fig. 4). Total genomic DNA from various OM

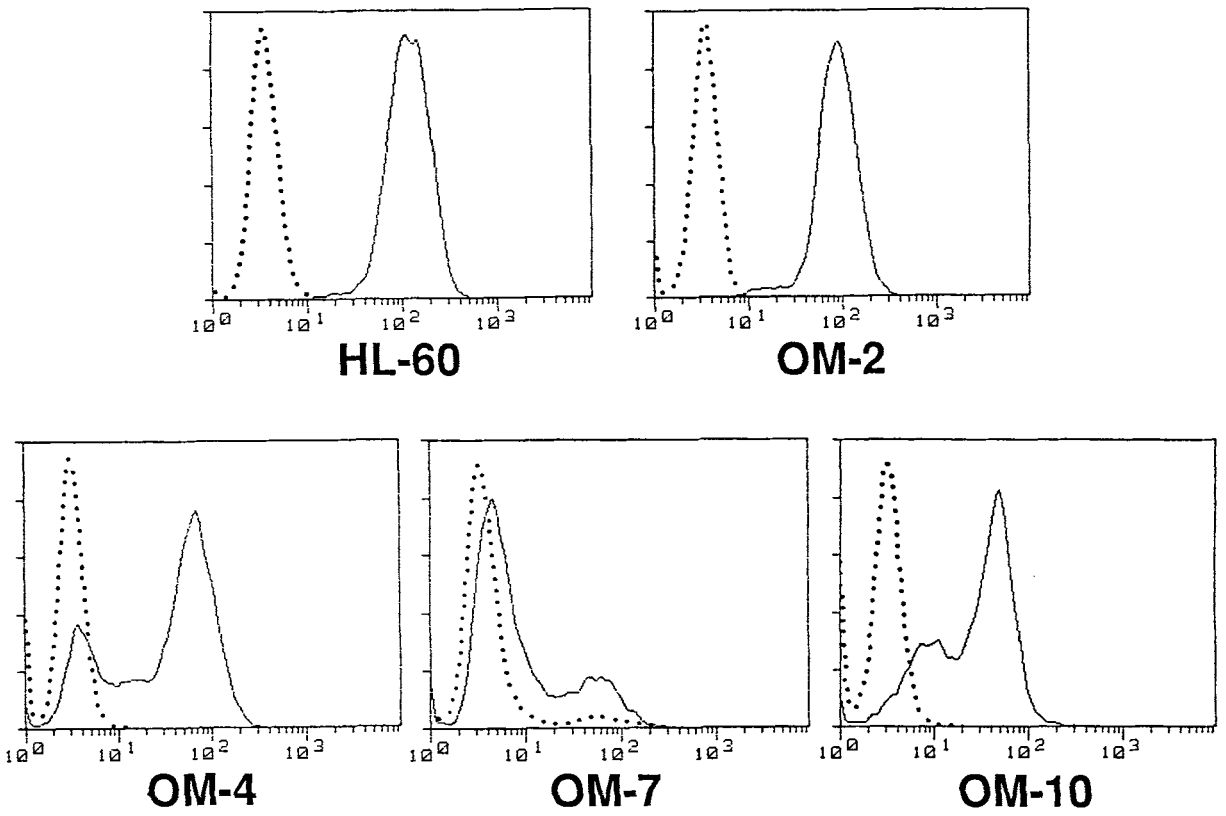

Fig. 2. CD4 surface expression by HL-60 and OM cultures during various stages of HIV-1 expression. Histograms from flow cytometric analysis are presented that demonstrate the reactivity (as relative log fluorescence intensity) with a CD4-specific monoclonal antibody (OKT4) (solid line) for each cell culture. A CD8-specific monoclonal antibody (OKT8) (dotted line) was used as a negative control. In these experiments, staining of cells with either OKT4 or OKT4A gave identical results.

culture stages was restricted for an internal HIV-1 Eco-RI/Bam-HI 2.7 kb fragment and probed with a corresponding Eco-RI/Hind-III 2.4 kb fragment of p-BENN 6 [8]. The 8E5 T-cell clone was used as a positive control for a single integrated HIV-1 provirus per cell [11] and produced a strong band of 2.7 kb. From OM-2 cell lysates, no detectable HIV-1 fragment was observed, indicating that a low percentage of OM-2 cells harbored the HIV-1 genome. Southern hybridization results from OM-7 and OM-10 cell lysates reflected their predicted level of HIV-1 expression. OM-7 lysate produced an HIV-1 band of the correct molecular size that was several fold stronger than the one copy per cell control. OM-10 lysate produced a very weak band at the same position, indicating fewer than one HIV-1 proviral copy per cell. The HIV-1$^+$ OM-10 subclone, OM-10.1, also produced an intense band of 2.7 kb.

Extremely Low Frequency of HIV-1 Infection Among OM-2 Cells

Because of the absence in OM-2 lysates of an HIV-1 genomic band by Southern analysis, an additional subcloning of OM-2 cultures was performed to determine the percentage of cells capable of spontaneous HIV-1 expression. Over a 3-month period, none (0/48) of the expanded OM-2 subclones displayed any indication (by RT, IFA, or p24 antigen) of HIV-1 expression (data not shown) indicating that the activation event must occur in less than 1 in 50 cells.

Since single cell cloning did not produce any HIV-1-expressing subclones (out of 48 clones tested), $\log_{10}$ dilutions of OM-2 cells were cultured to determine the minimum number of cells required to achieve spontaneous HIV-1 expression (Table I). Cultures of OM cells containing 10^6 or 10^5 cells per culture were capable of spontaneous HIV-1 expression with the same

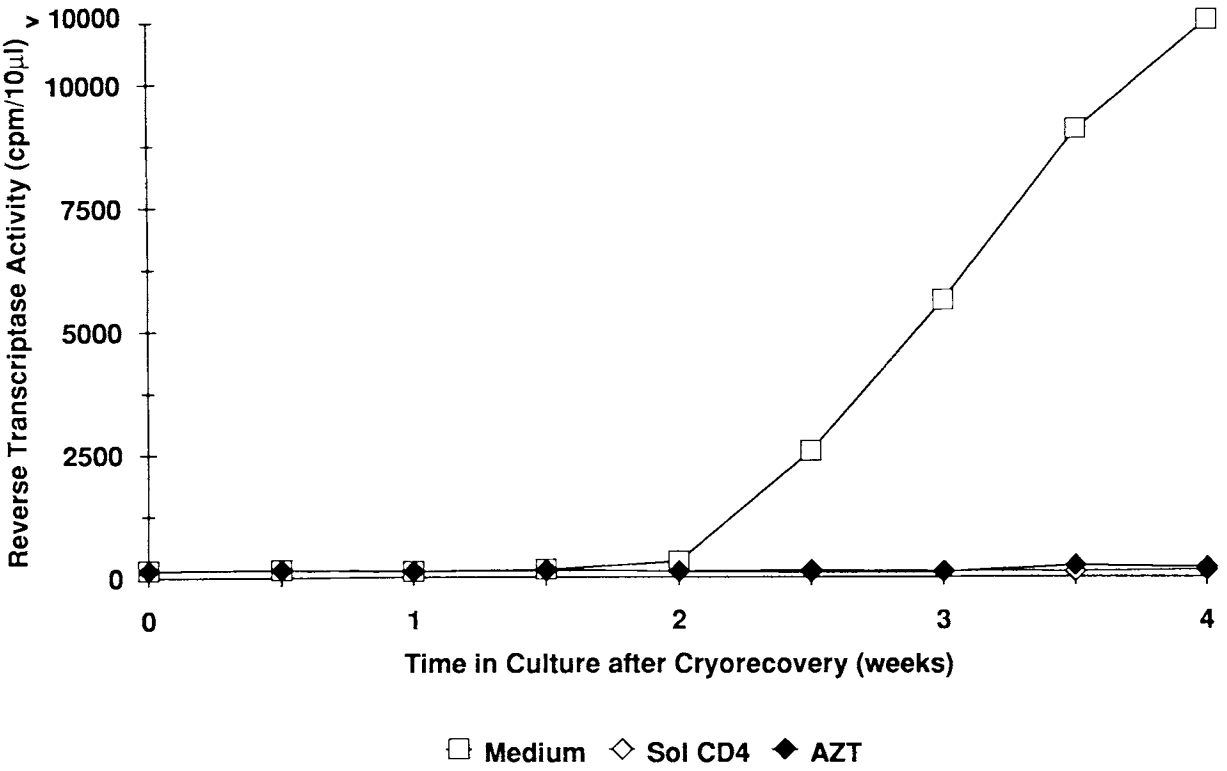

Fig. 3. Reverse transcriptase analysis of OM-2 cultures maintained with inhibitors of acute HIV-1 infection. Cryorecovered OM-2 cells were divided into separate cultures and maintained in medium (square symbols) or in the presence of either recombinant soluble CD4 at 20 μg/ml (open diamond symbols) or AZT at 10 μg/ml (closed diamond symbols) for a period of 4 weeks. Cells were diluted at 1:10 every 72 h and serial culture supernatants were collected. RT activity of supernatants was determined simultaneously. Data are representative of two separate trials.

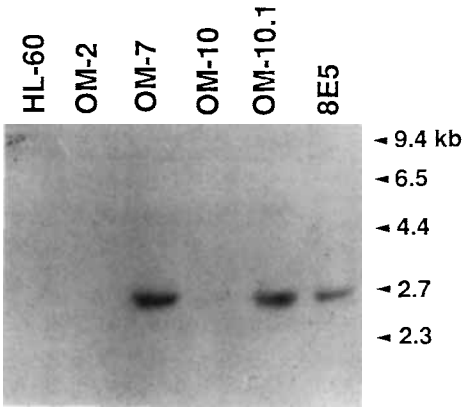

Fig. 4. Southern blot analysis of total cellular DNA derived from HL-60, 8E5, and progressive stages of OM cultures. Eco-RI and Bam-HI digestion produce an internal HIV-1 fragment of 2.7 kb which is very evident in 8E5, OM-7, and OM-10.1 lanes. A band at the same position is faint but evident in the OM-10 lane. No other bands were seen on the autoradiograph. Except for the 2.7 kb band indication, the molecular weight markers are based on the migration of lambda phage DNA restricted by Hind-III digestion.

kinetics as an undiluted OM-2 population ($>2.5 \times 10^6$ cells). However, OM cultures established with 10^4 cells per culture or less never underwent spontaneous HIV-1 expression and remained completely HIV-1$^-$ (by IFA) during the duration (6 weeks) of the culture period.

The frequency of OM-2 cells harboring the HIV-1 genome was further evaluated by PCR (Fig. 5). OM-2 cells were serially diluted into uninfected HL-60 cells and the total cell pellet was lysed and amplified for a region of the HIV-1 *gag* sequence. As a positive control, a similar cell dilution was performed on 8E5 cells. A specific PCR product was identifiable with 8E5 cells at the single cell level. However, the positive PCR signal was lost from OM-2 lysates after the 1.5×10^3 cell titration, confirming that less than 1 in 1,000 OM-2 cells contained the HIV-1 genome. In light of the clonal derivation of the OM line and the extremely low frequency of cells harboring the HIV-1 genome, an extrachromosomal HIV-1 genome must have existed during OM clonal expansion. The unintegrated HIV-1

TABLE I. HIV-1 Expression by Cultures Derived by Log Dilution of OM-2 Cells

Weeks postculture	Number of OM-2 cells per culture							
	$>2.5 \times 10^6$	10^6	10^5	10^4	10^3	10^2	10	1
0	–[a]	–	–	–	–	–	–	–
1	–	–	–	–	–	–	–	–
2	+ (5%)	+	+	–	–	–	–	–
3	+ (25%)	+	+	–	–	–	–	–
4	+ (75%)	+	+	–	–	–	–	–
5	+ (75%)	+	+	–	–	–	–	–
6	+ (25%)	+	+	–	–	–	–	–

[a]Data are presented from direct immunofluorescence assay for cellular HIV-1 expression with "+", the percentage of cells shown in parenthesis were HIV-1$^+$, and "–", all cells examined (>500) were HIV-1$^-$. The percentage of HIV-1$^+$ cells in the 10^6 and 10^5 cultures were equivalent to or greater than the undivided OM-2 culture ($>2.5 \times 10^6$ cells) at each time point.

DNA apparently was nonfaithfully transmitted during cell division but retained the capacity to generate a classical acute infection of susceptible HL-60 cells in culture.

DISCUSSION

In this report we characterize an HIV-1-infected promyelocytic cell line, OM, and describe its unique pattern of HIV-1 expression. Although derived from a biologic clone, OM cultures undergo an acute spreading HIV-1 infection which initiates from a very low frequency of cells harboring an extrachromosomal HIV-1 genome. The addition of either AZT or recombinant soluble CD4 completely prevented transmission of HIV-1 by OM cells. The inhibitory effect of AZT treatment in this system confirms an acute infection due to the inability of AZT to prevent expression from an integrated HIV-1 provirus [17]. The course of this spreading infection appeared identical to that of a classical acute HIV-1 infection, reaching a peak of expression and then declining. From the surviving cells, we were able to identify clonal populations which harbored integrated HIV-1 provirus.

Our conclusion of an unintegrated HIV-1 genome being responsible for the spreading acute infection of OM cultures is based on several lines of evidence. The dual subclonings of this line (with cloning efficiencies of 17.7% and 36%, respectively) and the extremely low frequency of cells harboring the HIV-1 genome indicates the presence of an extrachromosomal form of HIV-1. Clumping of infected and uninfected cells could result in a mixed population of cells falsely indicating a statistical clonal frequency. However, to account for <0.1% of the cells harboring an HIV-1 provirus, >1000 cells would have had to be cloned in a single well during both subclonings. An outgrowth of HIV-1-resistant cells during expansion for cryopreservation also cannot account for the initial low frequency of infected OM cells. If this were the case, a spreading acute infection of OM cultures would never occur because 99.9% of the cells would thus be resistant.

The mechanisms responsible for activation of the extrachromosomal HIV-1 genome and initiation of the acute infection remain conjectural. Owing to previous reports that unintegrated HIV-1 DNA is incapable of producing infectious progeny [3], it could be augured that integration is the necessary activating step in OM-2 cells harboring an extrachromosomal HIV-1 genome. The integration may require cellular activation signals, as demonstrated in resting T-cells where a partially reverse-transcribed HIV-1 genome can remain dormant until physiologic activation of the cell permits viral integration [18].

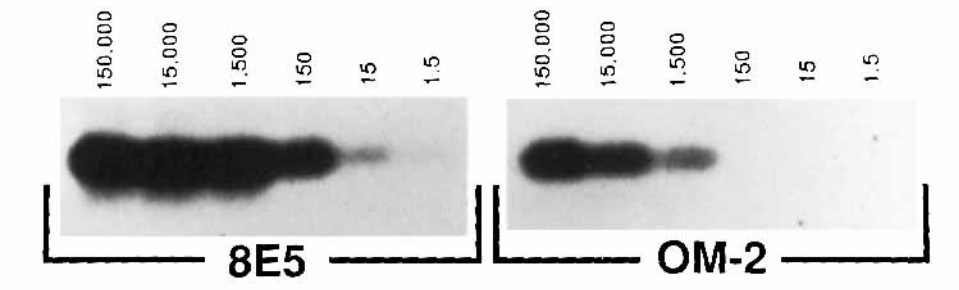

Fig. 5. PCR amplification of genomic DNA to determine the frequency of cells harboring HIV-1 genome. 8E5 (**left panel**) and OM-2 (**right panel**) cells were serially diluted from 150,000 to 1.5 cells with uninfected HL-60 cells. Specific products of PCR amplification for HIV-1 *gag* sequences were confirmed by Southern blotting; they are presented as an autoradiographic exposure. Longer exposures of the radioactive blot did not demonstrate a positive signal with OM-2 cells past the 1,500 cell dilution.

Alternatively, a cellular change may take place to permit a rapid production of infectious progeny off of the unintegrated HIV-1 genome. Along those lines, we have ruled out that spontaneous cellular differentiation accounts for activation of the extrachromosomal HIV-1 DNA. All stages of OM cultures were phenotypically and cytochemically similar to uninfected parental HL-60 cells when examined for spontaneous differentiation (Butera, manuscript in preparation).

Just as intriguing as the spontaneous HIV-1 expression in early OM cultures is the spontaneous reversion to viral dormancy of cultures maintained past the peak of HIV-1 expression. When OM-10 cultures were maintained for protracted periods the HIV-1-expressing subpopulation persisted, presumably due to stable integration of HIV-1 genome in those cells. The HIV-1$^-$ cells in these long-term cultures, although CD4$^+$, did not appear susceptible to reinfection. Several possible explanations could account for these observations. First, an overgrowth of infection-resistant cells, especially when coupled with a cytostatic/cytopathic viral effect, would account for the fall of viral positivity and the maintenance of HIV-1$^-$ cells in long-term OM cultures. However, a lack of cytopathicity among HIV-1-infected HL-60 cells has been a consistent observation [19,20] and may be related to a low level of CD4 expression by these cells [21].

Second, the extrachromosomal HIV-1 genome may be diluted from the culture by nonfaithful replication and only the cells which have stably integrated the HIV-1 provirus can continue expression. Such a possibility would require a transcriptionally competent, unintegrated form of HIV-1 DNA to be transiently maintained in OM cells. Furthermore, an alteration of CD4 would have to accompany its return to the cell surface after transient HIV-1 expression and prevent subsequent reinfection of these cells. While both of these requirements seem unlikely, we are currently conducting experiments to examine the integrase function and circular HIV-1 DNA forms from these cells.

Progenitor cells in general may be natural hosts for alternative HIV-1 genomic replication. HL-60 cells naturally harbor episomal forms of certain proto-oncogenes, which are nonfaithfully transmitted and maintained as a function of time in culture prior to random integration [22]. Furthermore, as shown with other monoblastoid cells [6,23], HL-60 promyelocytic cells may be more permissive to an alternative viral life-cycle. Others have noted an instability of HIV-1 infection in HL-60 cells [19], which may be related to the alternative viral life-cycle that we are describing.

The OM cell line and subclones derived from it appear to be unique reagents for in vitro studies of several different aspects concerning HIV-1 propagation. This cell line can contribute to studies on the molecular mechanisms controlling HIV-1 integration and transmission during normal cellular division.

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