Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 9996–10000, November 1992
Medical Sciences

Productive nonlytic human immunodeficiency virus type 1 replication in a newly established human leukemia cell line

(differentiation/biphenotypic markers/infection/phorbol 12-myristate 13-acetate)

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Communicated by Edwin D. Kilbourne, July 10, 1992

ABSTRACT **We have isolated a lymphoid cell line, MDS, from the pleural exudate of a patient with chronic myelomonocytic leukemia. The cells are biphenotypic, containing various T-cell and myeloid markers, and are surface negative for CD4 and CD8 but have low CD4 mRNA. The cells grow in suspension with a doubling time of 15 hr, have been karyotyped as trisomy 21, are negative for human immunodeficiency virus type 1 (HIV-1), and are tumorigenic in the nude mouse. We have isolated two stable HIV-1-producing cell lines, MDS-T, by transfecting MDS cells with pHXBc2, and MDS-I, by infecting MDS cells with HIV-1_{IIIB}. In 24 hr, 1×10^5 MDS-T or MDS-I cells produce 46 ng of p24 per ml and reverse transcriptase that is capable of incorporating 0.2 pmol of [^{32}P]TTP into oligo(dT)·poly(A). Ultrastructural studies showed numerous mature viral particles in MDS-T and MDS-I cells that are capable of infecting T cells. HIV-1 infection could be inhibited by 25% in the MDS cells with the anti-CD4 antibody Leu 3a. For over a year MDS-T and MDS-I cells have been producing high concentrations of HIV-1 in culture. A subclone derived from the MDS cells behaves like the parent cells when transfected or infected with HIV-1. In contrast to other T-cell lines, neither phorbol 12-myristate 13-acetate nor tumor necrosis factor α stimulated the replication of HIV-1, whereas bromoadenosine 3′,5′-cyclic monophosphate or interferon α caused 50% and 80% inhibition of reverse transcriptase production, respectively. These chronically infected T-cell lines are a useful model system to study the effect of anti-HIV agents and cellular factors required for HIV-1 replication.**

The human immunodeficiency virus type I (HIV-1) has been designated as the etiologic agent of the acquired immunodeficiency syndrome (AIDS) (1–6). HIV-1 gene expression and replication are regulated by viral-encoded proteins and cellular regulatory proteins (7, 8). HIV-1 infection leads to an immunodeficiency state that may require many years before the characteristic pathology develops (9). To evaluate the latency in HIV-1 infection, several experimental models have been developed utilizing human cell lines of lymphoid and nonlymphoid origin (7, 10–12). Most T-cell lines that are infected with HIV-1 undergo lysis after several days but some are resistant to this cytopathic effect (4, 13–15). To evaluate the role of various cellular factors and the effect of antiviral agents on the replication of HIV-1 in T cells, it is crucial to have cell lines that can be infected and support nonlytic HIV-1 replication for prolonged periods of time and that produce large amounts of HIV-1 as measured by the production of HIV-1 reverse transcriptase (RT) and p24 antigen. We have established two stable HIV-1-producing cell lines, MDS-T (transfected with a HIV-1 proviral clone, pHXBc2) and MDS-I (infected with the HIV-1_{IIIB} strain), from the MDS cell line, which was derived from a patient with chronic myelomonocytic leukemia in the course of myelodysplastic syndrome. These two T-cell lines, which are surface CD4 negative and continuously produce high amounts of HIV-1 without causing cell lysis, are useful to study the effect of various antiviral agents as well as cellular factors necessary for the replication of HIV-1.

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MATERIALS AND METHODS

Origin of the Cell Line. The patient was a 73-year-old male with myelodysplastic syndrome. He developed multiple cutaneous infiltrates diagnosed as lymphomas histologically. The MDS cell line was prepared from his pleural exudate. Cells were collected by centrifugation and grown in Eagle's minimal essential medium (MEM; GIBCO) with 10% fetal calf serum and antibiotics (penicillin and streptomycin, 100 units/ml). These cells are passaged twice a week with a concentration of 2×10^5 per ml. Cells were treated with phorbol 12-myristate 13-acetate (PMA), 8-bromoadenosine 3′,5′-cyclic monophosphate (Br-cAMP; Sigma), tumor necrosis factor α (TNF-α; 1×10^8 units/mg of protein, a gift of Anthony Cerami, The Picower Institute for Medical Research, Manhasset, NY), or interferon α_{A2} (Hoffmann–La Roche) as described in the text. The cytotoxicity of these agents on various cell types was determined by trypan blue dye exclusion as well as by propidium iodide staining followed by cytofluorographic analysis.

Nude Mouse Tumor. Each nude mouse was injected with 1×10^7 cells subcutaneously on both sides of the mouse. Tumor measurement was taken 3 weeks after injection and repeated every week.

Virus Propagation and HIV-1 Infection of MDS Cells. HIV-1_{IIIB} obtained from the AIDS Research and Reference Reagent Program, Rockville, MD, was grown in CEM cells, and the stock was standardized to contain equivalent amounts of virus based on RT activity (1×10^5 cpm/ml) and kept frozen at −70°C until used. One and a half hours after infection with HIV-1, MDS cells were washed three times with phosphate-buffered saline (PBS; pH 7.2) and cultured with fresh medium for several generations to isolate the MDS-I cell line. A similar infection protocol was used to infect CEM or freshly isolated T cells from peripheral blood lymphocytes with the

Abbreviations: HIV-1, human immunodeficiency virus type 1; MDS, cell line derived from a patient with myelodysplastic syndrome; MDS-T, cell line derived from transfection of MDS cells with HIV-1 proviral clone pHXBc2; MDS-I, cell line derived from infection of MDS cells with HIV-1_{IIIB} strain; RT, HIV-1 reverse transcriptase; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; TNF-α, tumor necrosis factor α; TCR, T-cell receptor; IL-2, interleukin 2; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; mAb, monoclonal antibody.

virus isolated from the MDS-I cell line. MDS cells were pretreated with the murine monoclonal anti-CD4 antibody Leu3a (30 μg/ml; Becton Dickinson) for 1½ hr prior to HIV-1 infection. The cells were then washed and infected with HIV-1_{IIIB} by the protocol described above to evaluate the role of CD4 mediated viral entry.

Flow Cytofluorometry for Surface Markers. Either control or HIV-1-containing MDS cell lines were characterized for cell surface phenotypes by two-color direct immunofluorescence assay with fluorescein isothiocyanate- [T-cell receptor (TCR) α or β, CD3, CD4, CD7, CD10, CD34, CD45, and CD71] and/or phycoerythrin- (CD8, CD13, CD15, CD20, CD25, CD33 and HLA-DR) conjugated monoclonal antibodies (mAbs) (Becton Dickinson) directed against T, B, and myelocytic cells. MDS or MDS-T cells (5×10^5) were stained with 20 μl of specific pairs of mAbs for 20 min at room temperature prior to washing and fixation. Flow cytographic analyses were performed on at least 2×10^4 cells per mAb set using a FACStar II (Becton Dickinson). Discrimination of subsets was obtained by multiple standards for calibration and by use of Simulset Software. Two-color parameter data were displayed as contour maps for numerical integration and determination of the percentage of cells positive or negative for fluorescence.

Plasmids and Transfections. The HIV-1 proviral clone pHXBc2 (16), a gift from Craig Rosen (Roche Institute of Molecular Biology, Nutley, NJ), was transfected into MDS cells by a modified DEAE-dextran method (17). After transfection, MDS cells were grown in MEM with 10% fetal calf serum for several generations to establish the MDS-T cell line. Viruses isolated from MDS-T cells were used to infect CEM or freshly isolated T cells from peripheral blood lymphocytes. The −671 HIV-1 long terminal repeat chloramphenicol acetyltransferase (LTR CAT) plasmid has been described elsewhere (18). The CAT assay was performed as described (17).

Viral Assays. The secretion of p24 antigen was determined by using a HIV-1 p24 core profile ELISA kit from NEN/DuPont (no. NEK-060) as recommended by the manufacturer. The amount of p24 was expressed in ng/ml of culture supernatant, which was properly diluted to determine the p24 values in the linear range. RT activity present in the culture supernatant was determined according to Hoffman *et al.* (19) with some modifications. Briefly, 10 μl of cell-free culture supernatant was used in 50 μl of reaction mixture containing 50 mM Tris·HCl (pH 8.0), 5 mM dithiothreitol (Sigma), 5 mM $MgCl_2$, 150 mM KCl, 0.05% Triton X-100, 0.5 mM EGTA, 50 μg of poly(rA)·$(dT)_{12-18}$ per ml (Pharmacia LKB), and 0.1 mCi of [α-^{32}P]TTP per ml (specific activity, 3000 Ci/mmol; 1 Ci = 37 GBq; Amersham). After 90 min of incubation at 37°C, 10 μl was spotted onto DE81 filter paper (2.4-cm circles, Whatman). The filters were then washed four times in 2× SSC (0.15 M NaCl/0.015 M sodium citrate, pH 7.0) at a volume of 100 ml per filter. The filters were then dried under a heat lamp for 10 min and assayed for radioactivity in 10 ml of Betafluor (National Diagnostics, Somerville, NJ).

Protein Kinase C (PKC) Assay. PKC assay from the MDS cells was performed at various time points following treatment with PMA (20 ng/ml). Cells were washed twice with ice-cold PBS and pelleted by centrifugation. The pellet was dissolved in a buffer containing 50 mM Tris·HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA, 0.3% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg of leupeptin per ml, 1 μg of antipain dihydrochloride per ml (Boehringer Mannheim), 10 mM benzamidine, and 0.1% lubrol and then homogenized. For each PKC assay, 250 μg (concentration, 1 mg/ml) of cell homogenate and 1 μCi of [γ-^{32}P]ATP (specific activity, 3000 Ci/mmol) per sample was used. The assay was carried out by using a PKC enzyme assay system (Amersham no. RPN 77) for 15 min at 25°C as suggested by the manufacturer and radioactivity was determined.

Transmission Electron Microscopy. The MDS, MDS-T, and MDS-I cell lines were processed for transmission electron microscopy by fixing the cell pellets with a mixture of 2.5% glutaraldehyde/2.5% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 3 hr at 4°C, washing in the same buffer, cutting into small pieces, and postfixing with 1% osmium tetroxide for 1 hr. They were then dehydrated in graded ethyl alcohol and embedded in Spurr's low-viscosity medium. Thin sections were cut on an ultramicrotome, stained with uranyl acetate and lead citrate, and viewed under a Phillips EM-400 electron microscope.

Northern Blot Analyses. The poly(A)$^+$ RNA extracted from MDS cells was blotted onto a nitrocellulose filter and hybridized with a CD4 probe isolated from T4-pMV7 plasmid (20) using a standard protocol (21). Similarly, the RNAs from MDS-T or MDS-I cells blotted onto nitrocellulose filters were hybridized with an antisense RNA probe prepared from the cDNA clone pGM93 (ref. 22; obtained from the AIDS Research and Reference Reagent Program, which was a gift of John Rossi) and are complementary to nucleotides 8475–9576 of the HIV-1 HXB2 GenBank sequence.

RESULTS AND DISCUSSION

Characterization of the MDS Cell Line. The MDS cell line was established from the pleural exudate of a patient with chronic myelomonocytic leukemia. These cells grow in suspension with a generation time of 15 hr and are karyotyped as trisomy 21. Immunophenotypic analyses revealed the presence of T-cell markers (CD3, CD7, and CD71) and a myeloid marker (CD15) indicating biphenotypic expression. These cells have immature T-cell characteristics since they lack surface CD4, CD8, and interleukin 2 (IL-2) receptor (CD25) but do express CD3 and TCR α and β. MDS cells also do not express B cell or CALLA antigens. Treatment with PMA and Br-cAMP differentiates them toward the T-cell lineage, as indicated by the increase of CD3 and TCR α and β markers, increased expression of the IL-2 receptor, a 4-fold increase in IL-2 production (as measured by using an ELISA kit, T Cell Sciences, Cambridge, MA), and the concomitant reduction of CD34 and CD71 markers (Table 1). Although these cells are surface CD4 negative, Northern blot analyses revealed a low amount of CD4 mRNA (data not shown). The PMA treatment alone marginally increased TCR α and β and

Table 1. Immunophenotypic characterization of control and differentiated MDS cells treated with PMA and Br-cAMP

Cluster designation	Antibody	% positive cells	
		Control	PMA + Br-cAMP
CD45	Anti-HLe-1	99	96
TCR α or β	Leu 4	12	91
CD7	Anti-Leu 9	99	99
CD3$^+$ CD15$^-$	Anti-Leu 4$^+$	4	39
CD3$^+$ CD15$^+$	Anti-LeuM1	52	78
CD3$^-$ CD15$^+$		34	0
CD4	Anti-Leu 3a	0	0
CD8	Anti-Leu 2a	0	0
—	Anti-HLA-DR	0	0
CD25	Anti-IL-2 receptor	0	43
CD71	Anti-transferrin receptor	94	59
CD34	Anti-HPCA-2	81	65
CD20	Anti-Leu 16	0	0
CD10	Anti-CALLA	0	0
TdT	—	(+)	(+)

Cells (1×10^6 per ml) were treated with PMA (20 ng/ml) and Br-cAMP (1 mM) for 48 hr and then harvested for flow cytofluorometry. TdT, terminal deoxynucleotidyltransferase.

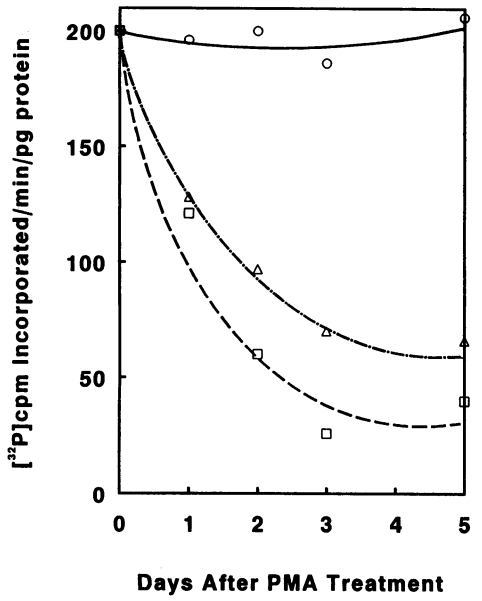

FIG. 1. PKC assay from the MDS cells at various time points following treatment with PMA (20 ng/ml). After treatment with PMA, cells were washed twice with ice-cold PBS and assayed for PKC. ○, Untreated control; △, 24 hr after PMA treatment monolayer cells were washed and the cells were harvested on the second, third, and fifth days; □, PMA was present during the entire period of experiment.

the CD3 antigen expression significantly but had no effect on CD4, CD25, CD34, or CD71 expression. Br-cAMP alone did not change the CD25, CD34, CD71, and TCR α or β but significantly increased the CD3-positive cell population. The detailed description of differentiation and surface markers of MDS cells will be published elsewhere.

Upon addition of 20 ng of PMA per ml, the MDS cells formed a monolayer within 30 min. After 4–5 days, however, they ceased to multiply. The attachment of the cells was not inhibited by cycloheximide, indicating that new protein synthesis is not involved in the process. A single exposure to PMA commits the cells to grow as a monolayer, since after trypsinization and replating (in the absence of PMA) they continue to grow for four or five generations as a monolayer. Since it is virtually impossible to wash out the PMA from the cells, we cannot exclude whether the residual PMA is responsible for the behavior of these cells, even after trypsinization and replating. In addition, PMA down-regulates PKC, and after 3 days PKC activity declined to 20% of the level of the untreated cells (Fig. 1).

The MDS cells are tumorigenic and, upon subcutaneous injection of 1×10^7 cells into nude mice, 2×2 cm tumors were formed within 2 months. A cell line was established from the tumor cells that proved to be genotypically identical to the original cells.

The MDS cells contain a factor that promotes transcription from the HIV-1 LTR. The expression of −671 HIV-1 LTR CAT plasmid is substantially higher in the MDS cells compared to the Jurkat cells but lower than in the HepG2 cells (17), which may indicate the presence of a small amount of "tat-like" trans-acting protein that is constitutively produced or due to the action of other transcription factors, such as SP1, AP1, NFκB, and others. Cotransfection of a *tat* gene construct with this plasmid in the MDS cells increased the LTR expression by about 30-fold (data not shown).

Establishment of HIV-1-Producing Cell Lines. Transfection and infection of the MDS cells were performed to establish chronic HIV-1-producing cell lines. Seven days after transfection of the MDS cells with pHXBc2, several thousand picograms of p24 and high RT activity were observed, consistent with viral replication. Cytofluorographic analysis using an anti-p24 murine mAb performed 3 weeks later showed that 50% of the cells were positive for p24 (data not shown). This high frequency of cells expressing p24 suggested the possibility of secondary infection after transfection. The MDS cells are infectable with HIV-1_{IIIB} even though they do not express surface CD4. We could, however, detect low levels of CD4 mRNA in these cells by Northern blot analysis (as described above), and anti-CD4 antibody (Leu 3a) inhibited HIV-1 infection by 25%, suggesting at least, in part, that viral entry could be mediated by CD4. The transfected (MDS-T) and infected cells (MDS-I) have been maintained stably in culture for over a year and passaged twice a week. In 24 hr, 1×10^5 transfected cells and infected cells produced about 46 ng of p24 per ml and RT that was capable of incorporating 0.2 pmol of [^{32}P]TTP into oligo(dT)·poly(rA) (Fig. 2). Electron microscopic studies revealed budding and secreted mature virions from MDS-T and MDS-I cells (Fig. 3). The secreted viruses from MDS-T and MDS-I (equivalent to 0.02 pmol of [^{32}P]TTP by RT assay) were capable of infecting the CEM T-cell line and freshly isolated T cells from human peripheral blood. We did not observe any cytopathic effect in either the MDS-T or MDS-I cells; however, their division time was only slightly slower than that of the parent MDS cells. No remarkable syncytia formation or ballooning was observed in these cells, the characteristics of which are common for other HIV-1-infected T cells. Northern blot analyses of mRNAs isolated from MDS-T cells showed all three (9.2, 4.3, and 1.8 kilobases) HIV-1-specific transcripts indicating active viral replication (Fig. 4). Similar results were obtained from MDS-I

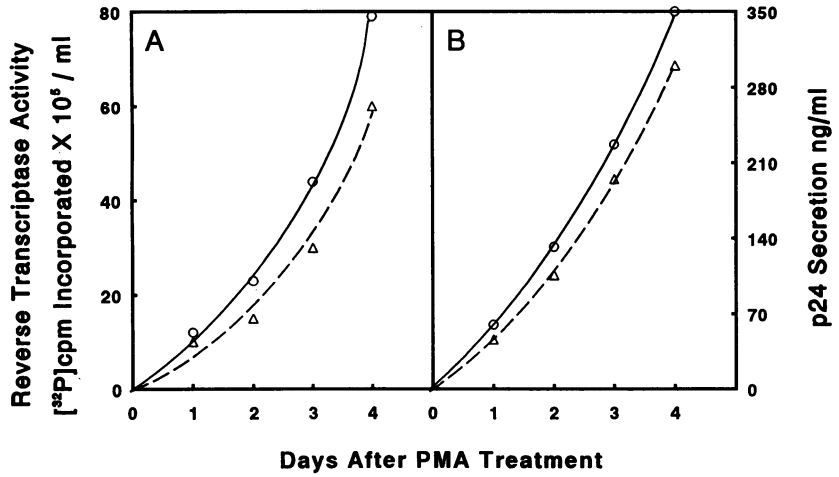

FIG. 2. Production of RT (*A*) and p24 (*B*) in control (△) and PMA-treated (20 ng/ml; ○) MDS-T cells at various time points.

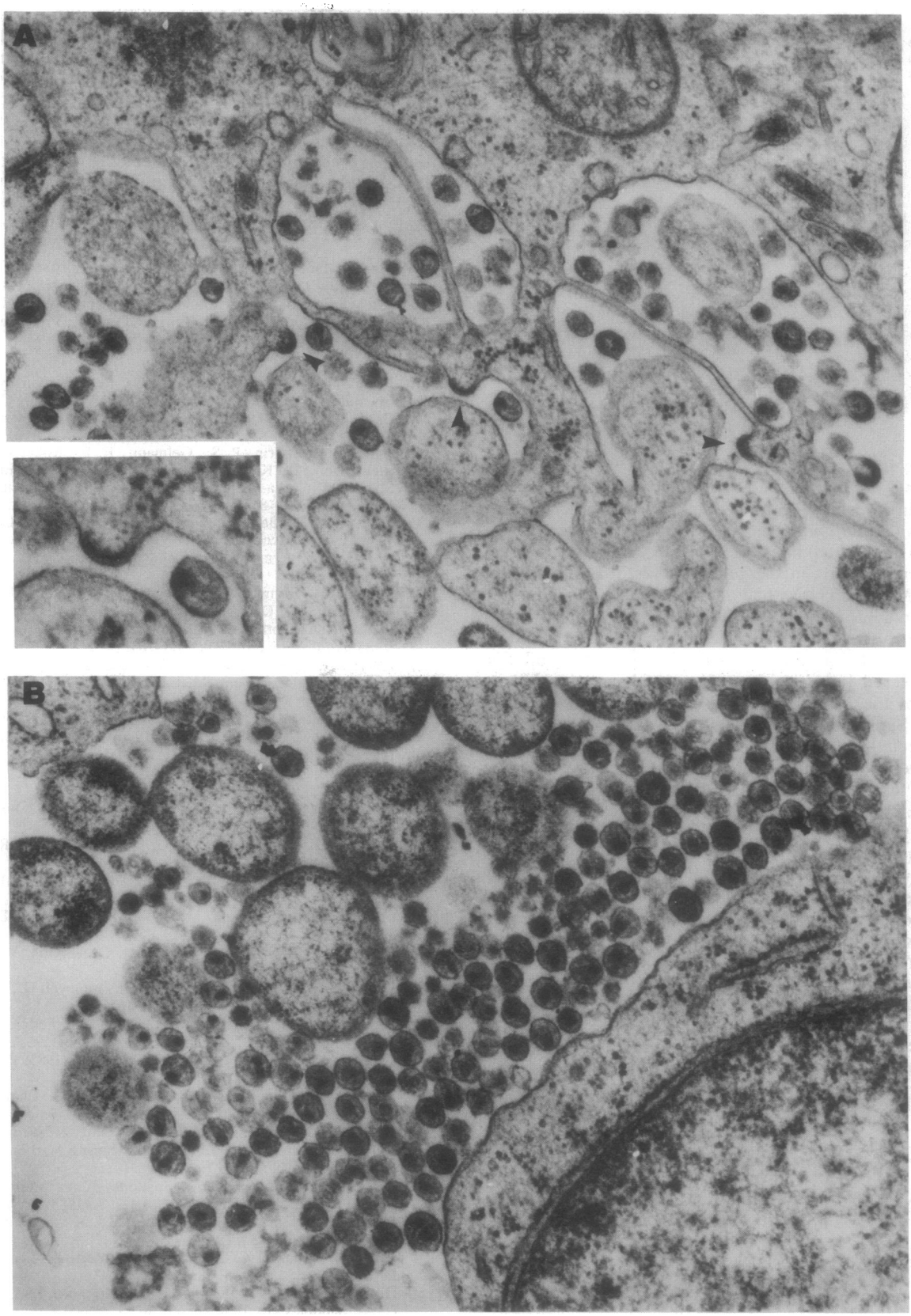

FIG. 3. Electron micrographs of the MDS-T (*A*) and MDS-I (*B*) cell lines showing numerous extracellular mature viral particles in the cytoplasmic vacuoles and extracellular spaces. Occasional budding of viral particles on the plasma membrane of the cells that resembled the morphology reported by other investigators (23, 24) was also noticed. The mature viral particles in the cytoplasmic vacuoles and in the extracellular spaces were round with a well-defined membrane and a variable dense central nucleoid present in both cell types. A dense crescent-shaped budding is shown on the nuclear membrane (*A*, *Inset*). These particles measured 75–150 nm in diameter. The morphology of the uninfected cell line MDS was similar to that of transfected or infected cell lines except for the absence of cytoplasmic vacuoles. No mature viral particles or immature budding particles were identified in this control cell line. (*A*, ×40,100; *B*, ×47,600.)

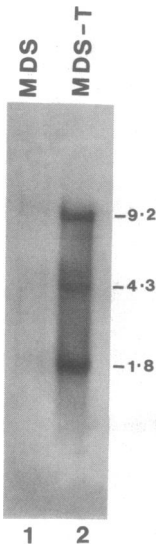

FIG. 4. Northern blot analysis of the mRNAs isolated from the MDS (lane 1) and MDS-T (lane 2) cells hybridized to an antisense probe generated from pGM93. Three HIV-1 transcripts (in kilobases) are indicated.

cells. Treatment of MDS-T and MDS-I with 50 units of interferon α per ml inhibited viral replication by about 80%, which is in agreement with the data reported earlier in other cell types (25, 26), whereas 10 μM Br-cAMP causes a 50% inhibition in both cell lines even though there are no cAMP-responsive elements in the genome of HIV-1. In contrast, neither PMA nor TNF-α had any remarkable effect on viral replication (Fig. 2 and Table 2).

To ensure that there was no clonal variation in the MDS cells, we isolated a subclone (MDS-C1) by limiting dilution that expressed lymphocytic and myeloid markers. Ninety percent of the MDS-C1 cells express the T and myeloid markers. Similar to the MDS cells, the MDS-C1 cells could be transfected with pHXBc2 and infected with HIV-1_{IIIB}, and 7 days after either transfection or infection the production of 1.9×10^6 cpm of RT per ml and 10 ng of p24 per ml was observed. The virus produced by MDS-C1 cells is capable of infecting CEM and freshly isolated T cells from peripheral blood lymphocytes (data not shown). Additionally, we did not observe any cytopathic effect of HIV-1 on MDS-C1 cells after either transfection or infection.

Three other nonlytic HIV-1-producing T-cell lines have been described, two derived from HUT-78 and the other derived from CEM cells (4, 13–15). In contrast to the MDS cells, however, these cell lines express surface CD4. The MDS cells are a useful model system to study HIV-1 replication in T cells since they are readily transfectable and infectable with HIV-1 and are stable in long-term culture producing large amounts of virus. In addition, viral replication in the MDS-T or MDS-I cell lines differs (Table 2) in response to some of the HIV-1-stimulatory agents, such as PMA or TNF-α, reported in other cellular systems (9, 18, 26–28). This finding suggests the presence or absence of a cellular factor(s) that does not permit a similar pattern of HIV-1 replication. In conclusion, we have established a lymphoid cell line with T and myeloid antigenic (biphenotypic) expression, which is transfectable and infectable with HIV-1 and capable of producing infectious virions without showing any cytopathic effect. This cell line is a useful model system to study the regulation of HIV-1 replication.

Table 2. Effect of TNF-α, PMA, Br-cAMP, interferon αA_2 (IFN-αA_2), or 3'-azido-3'-deoxythymidine (AZT) on expression of RT in MDS-T cells

Treatment	RT activity, cpm $\times 10^6$ per 10^5 cells per ml
None	2.4
TNF	2.3
PMA	2.1
Br-cAMP	1.2
IFN-αA_2	0.5
AZT	2.1

MDS-T cells were incubated for 48 hr with TNF-α (200 units/ml), PMA (20 ng/ml), Br-cAMP (10 μM), IFN-αA_2 (50 units/ml), and AZT (1 μg/ml). Data represent the average value of three or more experiments.

We thank Ms. M. Tenenbaum for electron microscopy and Ms. J. Carafa for flow cytofluorometry. This work was supported by The T. J. Martell Foundation for Leukemia, Cancer and AIDS Research. R.B. is recipient of National Institutes of Health First Award 1 R29 CA56302-01.

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