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13. ABSTRACT (Maximum 200 words) In the past year, we have made considerable progress in pursuing the specific aims of our proposal. We have investigated interaction of HIV with monocyte-macrophages. We have successfully identified responsive elements in the long terminal repeat (LTR) of HIV which interact with steroid hormone receptors that function as transcription factors. Importantly, we have characterized the differential response in monocyte-macrophages compared to T cells which depends upon expression of such receptors. In addition, we have begun to pursue transduction of monocyte-macrophages using the adenoassociated virus vector system for purposes of introducing anti-HIV gene.					
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FOREWORD

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Pathobiology of HIV in the Human Monocyte-Macrophage

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INTRODUCTION

The human immunodeficiency virus (HIV) infects circulating monocytes and tissue macrophages. Infection of this cell lineage is important with regard to pathogenesis of impaired host defense in patients with AIDS as well as in the neurological syndromes associated with HIV disease. Furthermore, monocyte-macrophages function to elaborate a variety of cytokines which can contribute to the inflammatory and wasting manifestations of HIV disease.

We have studied the interaction of HIV with monocyte-macrophages in our contract with the Department of Army. Our initial work focused on dysregulated cytokine production of HIV infected monocyte-macrophages. Our current work addressed transcriptional regulation of HIV within the monocyte-macrophages by steroid hormone receptors as well as strategies to deliver genes capable of inhibiting HIV using newly developed vectors. The methodology for the work performed was presented in the proposal (Section 4, "Description of Proposed Research"). The cell biological and molecular biological experiments focused on transcriptional regulation of HIV as well as characterization of functional genes. The development of antisense constructs within the adenoassociated virus vectors is an extension of the proposed work to characterize the functional genes of HIV and their effects in the monocyte-macrophage.

BODY

We extended several interesting observations with regard to interactions of steroid hormone receptors with the HIV negative regulatory element (NRE). By studying sequences of a wide variety of HIV isolates, it appeared that HIV strains obtained in Africa lacked the steroid responsive motif while all strains examined to date in Europe and North America contained the motif. This suggested that there may have been revolutionary drift in the transcriptional regulation of virus over time.

We also noted that monocyte-macrophages at different stages of differentiation had a range of expression of these steroid hormone receptors as detected by Northern blot. There was a clear

correlation between the modulation of HIV replication within a monocytic or lymphocytic cell line and the degree of expression of the receptor. Studies using the ligands for the RAR- α and RXR- α receptors, transretinoic acid and 9-cis-retinoic acid respectively, confirmed that these steroids worked specifically through their receptors and not through non-specific effects.

We have also tested the capacity of the adenoassociated virus vectors to transduce target monocytic cells. Permanent human monocytic cell lines such as THP-1 and U937 were facilely transduced by this vector. Initial studies in primary peripheral blood monocytes showed a very high transduction efficiency (greater than 50%) using reporter genes such as cat and luciferase. Pulmonary alveolar macrophages were not easily transduced (less than 5%) using the current vector stocks. It is possible that this is a problem with titer. We will therefore continue these studies attempting to transduce tissue macrophages in the form of alveolar macrophages by increasing the titer of vector. It is also possible that other promoters function better within the alveolar macrophage compared to the peripheral monocyte. The anti-HIV constructs which will be incorporated into these vectors have been developed in our laboratory as part of this program. They include anti-sense to TAT and VPR.

Thus in the past year's work, we have addressed viral and cellular factors which may regulate replication of HIV in different cell types. We have attempted to utilize information derived from this year and prior years of the program to provide a framework for therapeutic intervention.

CONCLUSION

Considerable progress has been made in the past year of our studies. We are optimistic that we will be able to utilize novel constructs for purposes of gene therapy that might reduce the cytopathic effects of HIV in monocyte-macrophages. In the context of the monocyte-macrophage, we aim to prevent expression of specific viral gene products that may contribute to pathogenesis in terms of cytokine elaboration and/or monocyte dysfunction.

Annual Report
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APPENDIX

1. Scadden DT, Wang Z, Groopman JE. Quantitation of plasma human immunodeficiency virus type 1 RNA by competitive polymerase chain reaction. J Infect Dis. 1992; 165:1119-1123.
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Quantitation of Plasma Human Immunodeficiency Virus Type 1 RNA by Competitive Polymerase Chain Reaction

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Clinical measures of human immunodeficiency virus (HIV) type 1 activity *in vivo* are limited and hinder the assessment of antiretroviral therapies. Reported here is a method for quantitating HIV-1 RNA in human plasma using the polymerase chain reaction (PCR). This method uses an internal cRNA standard generated from a cloned 113-bp deletion mutation of a highly conserved HIV-1 *gag* region sequence. The mutant cRNA (K4) was shown to amplify with efficiency equivalent to that of wild-type HIV-1. Known quantities of K4 cRNA added to wild-type HIV-1 in a competitive PCR strategy using a radiolabeled primer permitted quantitation of wild-type HIV-1 RNA over four orders of magnitude (10^3 – 10^6 RNA copies). RNA isolated from plasma from AIDS patients yielded 10^3 to 8×10^6 HIV-1 RNA copies/ml of plasma with an average intrasample coefficient of variation of .26. This method offers a sensitive assay with a broad dynamic range for monitoring HIV-1 activity in the plasma of AIDS patients. It may provide a useful tool for assessing the effects of antiretroviral therapy.

Currently available tools for measuring human immunodeficiency virus (HIV) type 1 *in vivo* are limited. Although circulating CD4⁺ lymphocyte cell numbers, serum HIV-1 p24 antigen, serum neopterin, serum β_2 -microglobulin, soluble CD8, and soluble interleukin-2 receptor levels have been proposed to be clinical indicators of HIV-1 disease activity, they are limited by sensitivity and specificity [1–6]. Quantitative plasma cultures and quantitative cultures of limiting dilutions of HIV-1-infected peripheral blood mononuclear cells (PBMC) are recently developed methods designed to better characterize the activity of the retrovirus [7, 8]. However, these methods are time consuming and expensive. Quantitation of HIV-1-infected PBMC by polymerase chain reaction (PCR) of PBMC DNA is a more rapid analysis and has been shown to correlate with the stage of the illness [9].

This method estimates the number of infected cells but may not be able to assess acute changes in viral replicative activity induced by drug therapies. Measurement of circulating virus is a potentially more sensitive assay for the activity of the virus.

Reasoning that HIV-1 plasma culture indicated the presence of intact virions in plasma, we undertook a study to evaluate PCR as a means of quantitating HIV-1 RNA in plasma. Due to the tube-to-tube variability of PCR final product observed by us and others [10], we sought to develop a competitive PCR-based method.

Methods

Patient samples. Blood (10–15 ml) was obtained by phlebotomy from HIV-1-infected individuals with an AIDS-defining diagnosis and from normal controls. Blood was collected in heparinized tubes (Becton-Dickinson, Rutherford, NJ), and plasma was harvested after centrifugation at 1200 g for 10 min. Plasma (2.0 ml) was mixed with 4.0 ml of 5 M guanidine isothiocyanate (Bethesda Research Laboratories [BRL], Gaithersburg, MD) and 100 μ g of yeast tRNA and was ultracentrifuged through 5.7 M cesium chloride (BRL) at 180,000 g for 18 h at 25°C. The resultant RNA pellet was resuspended in 0.3 M sodium acetate and ethanol-precipitated. The RNA pellet was washed with 70% ethanol and resuspended in water or stored under ethanol at –80°C.

All samples were obtained with informed consent from patients and handled in a protocol approved by the New England Deaconess Hospital Institutional Review Board.

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RNA standard. The HIV-1 *gag* region amplified by the oligonucleotide primer pair SK100/SK104 [11, 12] was amplified in the PCR protocol noted below. The resultant fragment was filled in using Klenow (BRL) and blunt end ligated into the plasmid pGEM-3Z (Promega, Madison, WI). A deletion mutation of 113 bp was produced, and the resultant plasmid (K4) was determined to be a satisfactory template for SK100/SK104-directed PCR by agarose gel electrophoresis. K4 cRNA was generated using T7 polymerase (Promega). The K4 cRNA was purified by ultracentrifugation through a 5.7 M cesium chloride cushion, precipitated, and resuspended at a concentration determined by both spectrophotometry and incorporation of [32 P]uridine triphosphate.

Reverse transcription and PCR. RNA was reverse transcribed in a 20- μ l volume containing 0.5 mM deoxynucleotides, 6 mM magnesium chloride, 50 mM TRIS (pH 8.3), 10 mM dithiothreitol, 200 units of recombinant Moloney murine leukemia virus reverse transcriptase (BRL), and 40 units of RNasin (Promega) at 37°C for 90 min. The reaction product (10 μ l) was

added to a 50- μ l final-volume solution containing 50 pmol each of SK100 and SK104, 0.1 pmol of 32 P-end-labeled SK104, 0.2 mM deoxynucleotides, 1.5 mM magnesium chloride, 50 mM potassium chloride, 10 mM TRIS, 0.1% gelatin, and 1 unit of Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT) and subjected to cycles of 92°C for 30 s, 55°C for 30 s, and 72°C for 1 min repeated 30 times with a 10-min extension at 72°C after the final cycle using a thermocycler (Ericomp, San Diego).

PCR product analysis. Amplified DNA was electrophoresed through 2% agarose or 6% acrylamide and visualized by UV light after ethidium bromide staining; the resultant bands were harvested and Cherenkov counted in a liquid scintillation counter (Beckman Instruments, Palo Alto, CA). Figure 1A summarizes the methods used.

Results

The HIV-1 *gag* region bracketed by the oligomers SK100 and SK104 was cloned and a deletion mutant produced (K4). K4 was amplified by the SK100/SK104 primer pair and yielded a PCR product readily distinguished from wild-type HIV-1 by either acrylamide or agarose gel electrophoresis (figure 1B, C).

The K4 cRNA was evaluated as a template for PCR using the SK100/SK104 primer pair and directly compared with the wild-type HIV-1 genome represented by RNA derived from HIV-1_{IIIIB}-infected H9 lymphoblastoid cells. After reverse transcription, 32 P-end-labeled SK104 was added to the PCR reaction mixture, and the PCR product was analyzed by electrophoresis. The yield of DNA was quantitated at different cycle numbers of PCR by the counts per minute present in the appropriate bands isolated from the electrophoretic gels. Figure 2A indicates that the rate of rise of incorporated 32 P-labeled primer is the same with either K4 or wild-type HIV-1_{IIIIB} as the PCR template. These data were corroborated using K4 compared with independently or concurrently (in the same tube) amplified known quantities of plasmid DNA of the HIV-1 clone HXB2 (provided by R. Gallo, National Institutes of Health, Bethesda, MD). K4 is amplified with an efficiency equal to that of wild-type virus and is therefore an appropriate template to use in a quantitative assay for wild-type HIV-1.

Having demonstrated that K4 can be readily distinguished from wild-type HIV-1 and yet can function as an equivalent target in PCR, we tested the sensitivity and range of our method of analyzing for HIV-1. Known amounts of K4 cRNA were diluted and used as starting material for the reverse transcription and PCR reactions. The resulting incorporation of 32 P-labeled tracer SK104 primer for each starting concentration of K4 cRNA is shown in figure 2B. The intra-sample coefficient of variation (CV) was 0.5 to 2.6 for the samples with 10^2 to 10^6 copies of K4 cRNA, respectively. The sensitivity of the assay is limited to 10–100 copies of K4 in the starting material but the dynamic range of the assay is at least four orders of magnitude. Since the range of in-

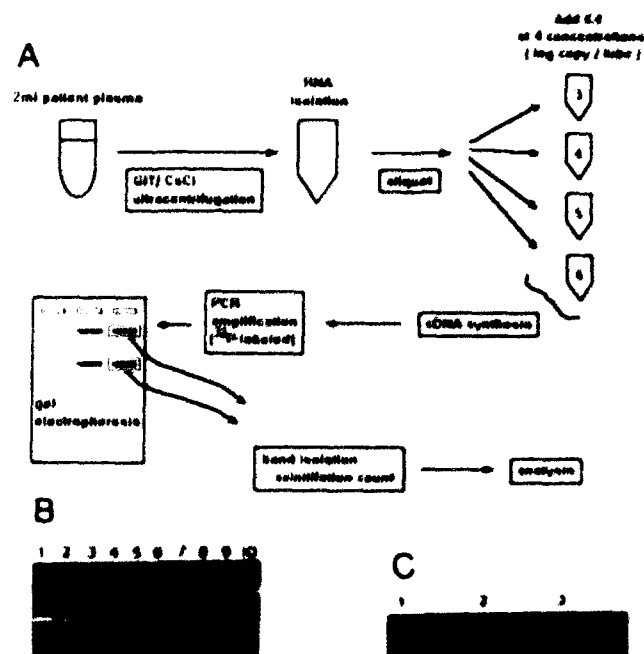


Figure 1. Summary of sample processing and analysis schema. GTT, guanidine isothiocyanate; PCR, polymerase chain reaction. Competitive PCR results of wild-type (wt) human immunodeficiency virus (HIV) type 1 (upper band) or K4 standard (lower band) at different ratios of wt to K4 (B) or at different dilutions of fixed ratio of wt to K4 (C). Fixed amount of K4 cRNA was maintained in samples corresponding to lanes 1–9 in B. Threefold dilutions of RNA from HIV-1_{IIIIB}-infected H9 cells were added sequentially from right to left in samples corresponding to lanes 10 through 2. Lane 1 contained only K4 cRNA (10^6 copies) and lane 10 contained only HIV-1_{IIIIB}-infected H9 RNA (1 μ g). C, Ratio of wt to K4 RNA represented by lanes 4 and 5 of B and intermediate ratio were subjected to sequential threefold dilutions before reverse transcription and amplification. Series of lanes for each numbered set (1–3) represent ethidium bromide-stained, UV-illuminated products of 30-cycle amplification.

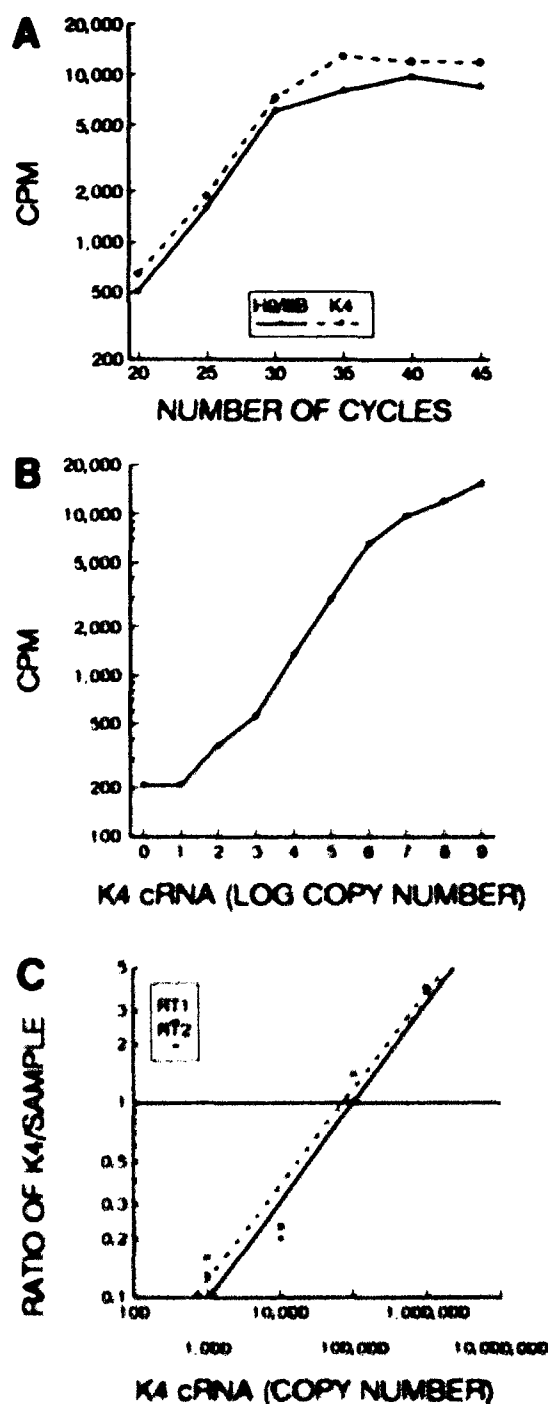


Figure 2. A, Equivalent amplification of wild-type (H9/H1B) or K4 RNA at different polymerase chain reaction (PCR) cycle numbers. H9/H1B and K4 cRNA (10^2 copies) were independently analyzed for incorporation of 32 P-labeled oligonucleotide primer (SK104) after reverse transcription and amplification by scintillation counting of isolated agarose gel slices of electrophoresed reaction products. B, Relationship of PCR product to quantity of input K4 cRNA. Known amounts of K4 cRNA were reverse transcribed and amplified for 40 cycles in presence of 32 P-labeled SK104 primer. Reaction products were subjected to agarose gel electrophoresis, gel slices were isolated, and scintillation counted. Number of

infectious virus detected in the plasma of HIV-infected individuals has been reported to be quite variable [13] and the range of virus particles containing detectable viral RNA (not necessarily infectious virus) may vary over a broader range, a wide dynamic range for the assay is desirable.

The relationship of end products of PCR to the starting quantities, in a competitive PCR strategy, is dependent on the ratio of the two templates at the time of initiation of the amplification protocol [10, 14]. At ratios of sample template to standard template of less than or greater than 1.0, the amplification of the two templates is not equal. Figure 1B represents PCR products of reactions containing a fixed amount of the K4 standard with increasing concentrations of sample HIV-1 RNA. The variable intensity of the visible lower band (K4) indicates the inconstant amplification of the K4 template in the presence of different concentrations of competing wild-type HIV-1 genome.

To investigate whether the differential amplification of wild-type versus K4 template was due to a limiting concentration of substrate, dilutions of RNA of three fixed ratios of wild-type to K4 (wt/K4) were added to the PCR reaction mixture and amplified (figure 1C). Dilution of the starting concentration of RNA did not alter the relationship between the wild-type and K4 final products, differential amplification of the two RNAs occurred unless wt/K4 approached 1.0.

Having demonstrated that the starting wt/K4 must approach 1 for equal efficiency of amplification, 2-ml human plasma specimens were processed, and four 10-fold dilutions of K4 cRNA were added to a fixed amount of plasma RNA ($0.5 \text{ ml } 10^2$ copies to $0.5 \text{ ml } 10^6$ copies). These dilutions were reverse transcribed and amplified, and the resultant products were analyzed as above. At equivalent levels of incorporation of radiolabeled primer the input wt/K4 was considered to be 1. The quantity of input wild-type RNA could thereby be extrapolated and the number of HIV-1 RNA molecules derived.

To verify the reproducibility of the assay, duplicate samples from a single phlebotomy were independently processed and analyzed. The results of one of these analyses are shown in figure 2C, and the results of 11 samples from 11 patients analyzed in this manner are reported in table 1. The average intrasample CV for 11 samples from 11 patients analyzed was 26 ± 15 .

To confirm that our results did not reflect cellular HIV-1, 40 cycles of PCR were done on patient samples without first submitting the samples to reverse transcription. No ampli-

K4 cRNA indicated is log of copies per assay. C, Analysis of one AIDS patient's plasma sample divided at time of phlebotomy and analyzed independently. Copy number of K4 cRNA molecules corresponding to K4-to-sample ratio of 1 estimates copy number of human immunodeficiency virus type 1 RNA in 1 ml of plasma ($8.6 \pm 1.4 \times 10^2$).

Table 1. Patient characteristics and results of studies of plasma samples from AIDS patients.

Sample no.	HIV RNA ($\times 10^3$ copies/mm ³ of plasma)	CD4 cells/ mm ³	Diagnoses	Anti- retroviral
1	7.75 $\pm$ 1.95	42	Crypto, CMV, KS	ZDV
2	1.3 $\pm$ 0.55	10	PCP, CMV, KS	ZDV
3	1.0 $\pm$ 0.1	20	KS	ddI
4	15.6 $\pm$ 7.4	30	CMV, KS	None
5	60.5 $\pm$ 9.5	30	Crypto, NHL	None*
6	82.5 $\pm$ 17.5	110	KS	None*
7	6.7 $\pm$ 3.0	40	KS	ddI
8	5.1 $\pm$ 0.7	20	KS	ddI
9	65.0 $\pm$ 0.1	20	KS	None
10	15.5 $\pm$ 3.5	200	KS	ZDV, IFN
11	31.5 $\pm$ 10.5	80	KS, NHL	ZDV

NOTE. HIV, human immunodeficiency virus; Crypto, cryptococcal meningitis or fungemia; CMV, cytomegalovirus; KS, Kaposi's sarcoma; PCP, *Pneumocystis carinii* pneumonia; NHL, non-Hodgkin's lymphoma; ZDV, zidovudine; ddI, didanosine; IFN, interferon- α .

* Drug withdrawn within 4 weeks.

fied products were seen, indicating that HIV-1-infected cellular DNA was unlikely to be present and therefore that cellular RNA could not account for our results. The specificity for HIV-1 of the bands assayed in this method was supported by the absence of bands in control plasma and by the same electrophoretic mobility of samples amplified with nonlabeled primers subsequently liquid-hybridized with a radiolabeled SK19 [12] probe. There were no false positives detected in 8 control plasma samples.

Discussion

Evaluation of the therapeutic benefit of medications in HIV-1 disease may be based on a number of clinical and laboratory parameters. Currently, the laboratory assays widely in use are indirect measures of the activity of the virus, such as T lymphocyte subsets, neopterin, or β_2 -microglobulin, or direct measurements of a viral product, such as HIV-1 p24 antigen. The latter assay is limited by the high frequency of p24 antigen-negative patients.

Improved direct measures of the infectious burden of HIV-1 are desirable because of the nonspecificity of the indirect laboratory parameters and because of the potential time delay for indirect measures to reflect alterations in circulating virus. Direct assays of the virus may permit more rapid analysis of anti-HIV-1 therapies. Methods that assess the circulating levels of free virus as opposed to virus incorporated into circulating cells may be particularly valuable because of the latency of HIV-1. A measure of free virus may be the preferred parameter to evaluate therapies directed at the replicative cycle of HIV-1, such as reverse transcriptase, tat or protease inhibitors, or inhibitors of virus assembly or release.

The method presented here uses common laboratory tech-

niques in conjunction with a mutant cRNA template to evaluate the quantity of HIV-1 RNA in the plasma of AIDS patients. This method requires a small quantity of plasma (obtained from a single phlebotomy tube of blood), which can be processed and the analysis completed within 48 h. An internal cRNA standard that is equivalently amplified in the PCR method is included in the analysis to avoid inaccurate quantitation of the sample material based on intrasample variability. Use of an external standard does not control for this variability, which may result in alterations in final product quantitation of up to 600% [10]. With an internal standard, the method presented here has an average intrasample variation of 26%.

In addition, the dynamic range of the assay is at least four orders of magnitude, substantially higher than those of other reported PCR-based strategies [13]. This feature of the assay makes it particularly attractive for the evaluation of antiretroviral therapies, where broad differences in circulating virus may be anticipated. The quantity of HIV RNA we have observed in AIDS patients has ranged from 10^3 to 8.3×10^4 copies/ml of plasma, which is comparable to other estimates of plasma viremia using PCR [13] or by extrapolating from circulating levels of HIV-1 p24 antigen [15].

The major limitations of the assay are the requirement for multiple dilutions of sample RNA (although we found that four dilutions are adequate in evaluating the AIDS population studied), the use of a radiolabel (though other methods of quantitation may sacrifice dynamic range), and issues of crossover contamination common to all PCR-based strategies. Methods of plasma collection were not found to influence our assay when we compared heparin, EDTA, and sodium citrate anticoagulants, perhaps because our method of RNA preparation reduces the presence of the anticoagulant in the PCR reaction mixture. The delay between sample collection and processing may be important. However, our preliminary observations suggest that there are no substantial differences in samples aliquoted on arrival in the laboratory (our laboratory is adjacent to the hospital where the samples were obtained) with one aliquot processed immediately and one processed after a 3-h delay, nor have we observed differences between samples processed immediately and plasma aliquots frozen at -80°C (unpublished data). Further definition of these parameters is in progress.

This relatively simple assay for quantitating plasma levels of HIV-1 RNA may provide a laboratory tool for measuring changes in circulating virus in vivo. The applicability of this assay to patients at different stages of HIV-1 disease and the correlation of viral burden with clinical stage are currently under investigation.

Acknowledgments

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Identification of a Novel Human Immunodeficiency Virus Strain Cytopathic to Megakaryocytic Cells

By Myriam S. Kunzi and Jerome E. Groopman

Impaired megakaryocytopoiesis may be a contributing factor to thrombocytopenia associated with human immunodeficiency virus (HIV) infection. Because HIV isolates differ in their host range and pathogenicity, we investigated whether HIV strains with demonstrable cell tropism and increased cytopathicity for megakaryocytes could be derived from the blood of thrombocytopenic HIV-infected individuals. We derived a strain, HIV-WW, from the peripheral blood of an individual with severe thrombocytopenia and found the virus to be highly and specifically cytotoxic to

CMK and DAMI megakaryocytic cells. CMK and DAMI cells were not permissive for the virus and HIV-WW induced cytopathicity for these megakaryocytic cells did not depend on viral replication. The CD4 N-terminus-binding domain of the HIV gp120 envelope protein did not appear to be involved in determining the cytopathic phenomenon. HIV may impair megakaryocytopoiesis through interactions at the cell surface in some cases rather than through viral entry and intracellular replication.

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THROMBOCYTOPENIA is a frequent finding in human immunodeficiency virus (HIV) infection. Antibody-mediated peripheral platelet destruction is well documented,^{1,3} but the clinical observation that platelet number often increases rapidly upon treatment with zidovudine (AZT)⁴ suggests that impaired thrombopoiesis, as a result of direct interaction of HIV with megakaryocytes, may be a contributing factor to the thrombocytopenia associated with HIV infection.⁵ About 25% of human bone marrow megakaryocytes have been found by flow cytometry to have detectable CD4 surface antigen,⁶ the primary receptor for HIV in T lymphocytes and monocytes.⁷⁻¹¹ The presence of viral genome in bone marrow megakaryocytes of some HIV-infected patients has been reported¹² and the virus can be incorporated in these cells in vitro.¹³

The recent availability of permanent cell lines of megakaryocytic lineage, such as the CMK^{14,15} and DAMI¹⁶ cells, has provided an in vitro system that facilitates the study of HIV infection in megakaryocytic cells. Although these cells were shown to be poorly susceptible to infection with HIV-1 IIIB, they were found to be readily and productively infected with HIV-2 ROD.¹⁷ Because HIV isolates differ in their host range¹⁸⁻²⁰ and their pathogenicity,²¹⁻²³ the question arises as to whether HIV-infected thrombocytopenic patients may harbor viral isolates with tropism or increased cytopathogenicity for megakaryocytes. To address the issues of tropism and cytopathicity of HIV in megakaryocytes, we studied the infectivity, cytopathic effects, and cellular tropism of HIV-WW, a virus derived from an asymptomatic patient with severe thrombocytopenia. We

found that HIV-WW was specifically cytotoxic to megakaryocytic CMK and DAMI cells but not to T-lymphocytic or monocytic cells. The cytotoxic effect on both CMK and DAMI cells did not require HIV-WW replication, suggesting a cytopathic effect at the megakaryocyte cell surface.

MATERIALS AND METHODS

Cells and virus The DAMI cell line, derived from the peripheral blood of a patient with megakaryoblastic leukemia,¹⁶ was a gift from S. Greenberg (Brigham and Women's Hospital, Boston, MA). These cells were maintained in Iscove's modified Dulbecco's medium (IMDM; GIBCO Laboratories, Grand Island, NY) with 10% heat-inactivated horse serum (HS; GIBCO), penicillin (P; 25 U/mL), streptomycin (S; 250 µg/mL; GIBCO), and L-glutamine (G; 2 mmol/L; Mediatech, Washington, DC). The CMK cell line,^{14,15} derived from a child with megakaryoblastic leukemia, was a gift from T. Sato (Chiba University, Japan). H9 (HUT78) cells^{24,25} were originally obtained from R.C. Gallo (National Cancer Institute, Bethesda, MD). U937 cells²⁶ were obtained from the American Type Culture Collection (Rockville, MD; catalog no. CRL 1593). CMK, H9, and U937 cells were maintained in RPMI 1640 (GIBCO), 10% heat-inactivated fetal bovine serum (FBS; GIBCO), and PSQ.

HIV-WW virus was obtained from an asymptomatic HIV-seropositive patient with a CD4⁺ cell count below 400 and persistent, severe thrombocytopenia. HIV-1 JR-CSF, isolated from the cerebrospinal fluid of a patient with acquired immunodeficiency syndrome (AIDS) dementia, was obtained from J.S.Y. Chen through the AIDS Research and Reference Reagent Program.^{27,28} HIV-RJ9435 was recovered from an asymptomatic HIV-seropositive patient with severe neutropenia. A stock of each virus was grown by coculture of infected peripheral blood mononuclear cells (PBMCs) with phytohemagglutinin (PHA)-stimulated PBMCs as previously described.²⁹ Virus was harvested in the supernatant, without concentration, when HIV p24 antigen levels reached a value greater than 500 pg/mL (HIV-WW, day 18 postinfection; HIV-1 JR-CSF, day 10 postinfection; HIV-RJ9435, day 20 postinfection).

Infections Cells were cultured in 24-well plates (Costar, Cambridge, MA). Cells (2×10^5 /well) were inoculated with 300 µL of virus stock, containing approximately 100 ng HIV p24 antigen. The viral inoculum was adsorbed to cells for 2 hours at 37°C. The cells were then washed with complete medium, plated at 1×10^5 cells/mL in fresh complete medium, and split 1:4 every 4 days. Mock infections were performed with 300 µL of supernatant from PHA-stimulated, uninfected PBMCs harvested after 4 days in culture. All infections were performed in duplicate and monitored by measuring HIV p24 antigen levels in the supernatant with a solid-phase antigen capture immunoassay kit (Abbott Laboratories, Chicago, IL).³⁰ Cell viability was assessed by trypan blue exclusion. In certain experiments, virus was depleted from the inoculum by fil-

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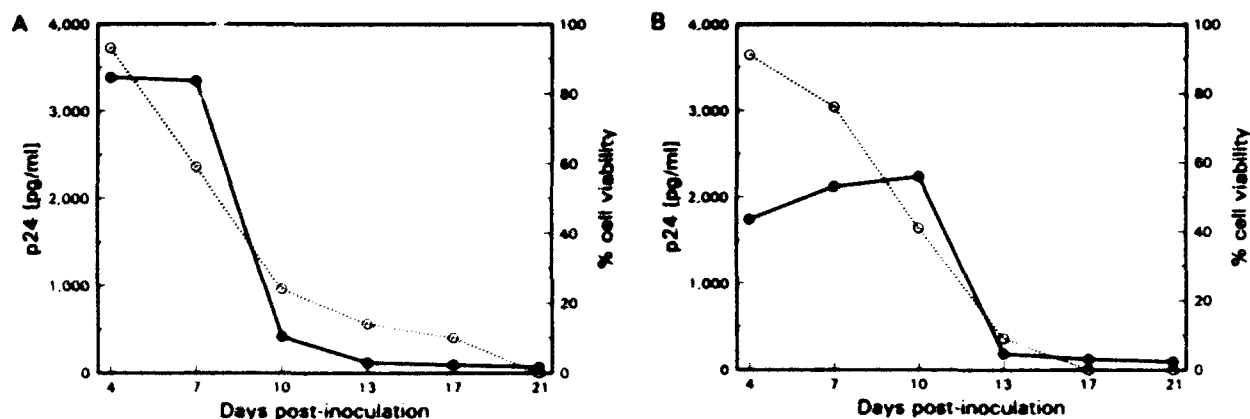


Fig 1. HIV-WW cytopathicity to CMK and DAMI cells. Cells (2×10^5) were challenged with HIV-WW (100 ng p24 antigen inoculum). Supernatant samples were harvested at the indicated times and assayed for viral p24 antigen. Viral p24 antigen levels are expressed in picograms per milliliter. Supernatant samples from mock-infected cultures were used as negative control. Cell viability was assessed by trypan blue exclusion. Each value represents the average of two independent measurements. This is one representative experiment of five independent experiments. (A) CMK cells. (B) DAMI cells. (○) Viability; (●) p24.

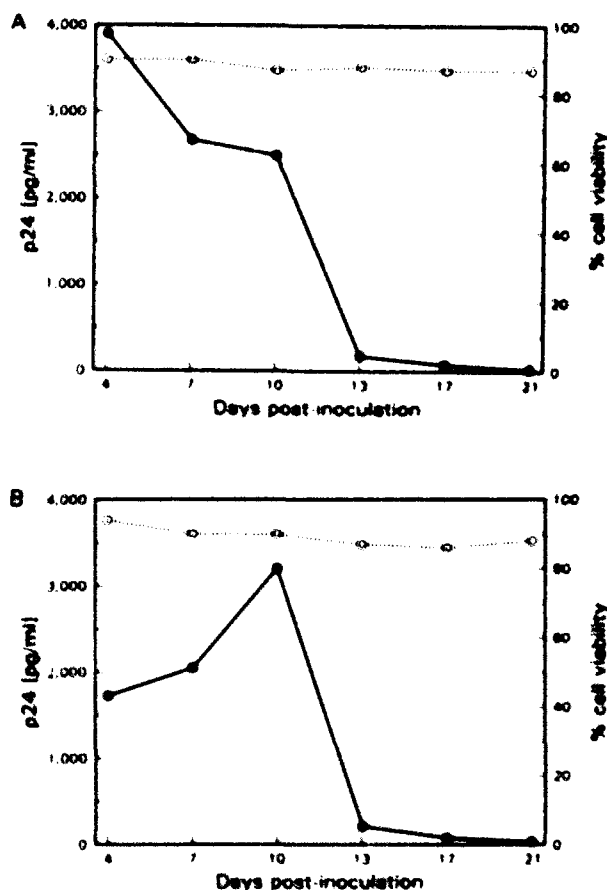


Fig 2. HIV-WW is not cytopathic to H9 (HUT78) or U937 cells. Cells were challenged with HIV-WW as described in Fig 1. Cell viability was assessed by trypan blue exclusion. Each value represents the average of two independent measurements. (A) H9 cell cultures. (B) U937 cell cultures. (○) Viability; (●) p24.

tration twice through a 0.2- μ m acetate cellulose filter (Sartorius, Goettingen, Germany). In the cultures in which serum from an asymptomatic HIV-seropositive donor was used to neutralize HIV-WW, 300 μ L of virus was incubated with 200 μ L of serum at 4°C for 1 hour before inoculation. Infection then proceeded as described above. In those cultures in which anti-Leu3A (Bect. n Dickinson, Mountain View, CA) was used to block the interaction of HIV with CD4, cells were incubated with dialyzed antibody at 5 μ g/mL at 37°C for 30 minutes before inoculation with virus. Infection was then performed as described above with the following modification: after adsorption, the cells were washed with, and plated in, complete medium supplemented with anti-Leu3A at 5 μ g/mL.

Polymerase chain reaction (PCR) analysis Total cellular DNA was extracted as previously described.^{11,12} HIV sequences were amplified by the PCR twice for 25 cycles, as previously described.^{11,12} using nested HIV gag-specific primer pairs. The first primer pair, SK100/SK104,¹¹ amplifies a 280-bp region of gag that is conserved among the HIV-1 isolates and HIV-2 ROD. The second primer pair, SK38/SK39,¹² amplifies a 115-bp region of gag conserved among the HIV-1 isolates. DNA isolated from HIV-1 IIIB-infected cells was used as a positive control and DNA extracted from mock-infected cell was used as a negative control. The specificity of the PCR products was verified by liquid hybridization to the 5'-end radiolabeled HIV-specific probe SK19.^{11,12} Amplification of β -globin genomic sequences^{11,12} was carried independently with the primer pair PCO3/PCO4¹¹ to assess DNA integrity and content. The sensitivity of the PCR assay was determined by dilution of chronically HIV-1-infected THP-1 monocytic cells with uninfected THP-1 cells. The assay is sensitive to 10^{-4} dilution of infected cells, corresponding to 100 HIV-1 infected cells in a sample of 10^6 cells per milliliter.^{11,12}

RESULTS

HIV-WW was recovered from an asymptomatic patient with severe thrombocytopenia and amplified by short-term coculture with PHA-stimulated PBMCs. To assess the tropism of this primary HIV strain, CMK, DAMI, H9, and

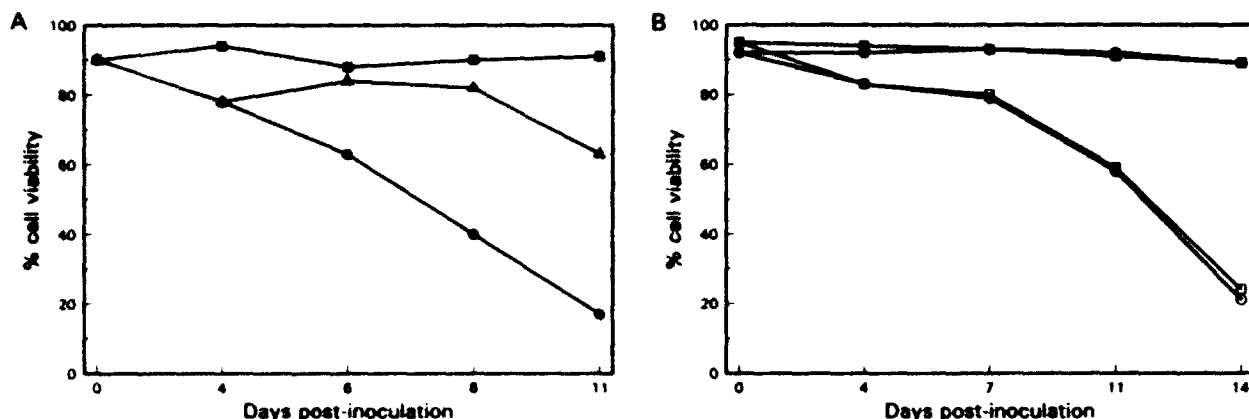


Fig 3. Cytopathicity is virally induced. (A) DAMI cells were challenged with 300 μ L PHA-stimulated uninfected PBMC supernatant (■), 300 μ L HIV-WW (100 ng p24 antigen) (●) or 300 μ L HIV-WW twice filtered through a 0.2- μ m cellulose acetate membrane to deplete the inoculum of virions (Δ). (B) CMK or DAMI cells were challenged with 300 μ L HIV-WW (100 ng HIV p24 antigen) (○ and □, respectively) or with 300 μ L heat-inactivated (56°C for 30 minutes) HIV-WW (● and ■, respectively). Cell viability was assessed by trypan blue exclusion. Each value represents the average of two independent measurements.

U937 cells were challenged at 2×10^5 cells/well with 300 μ L of virus stock containing 100 ng of HIV p24 antigen. HIV-WW was found to be cytotoxic to both the megakaryocytic Dami and CMK cells, but not to the T-lymphocytic H9 cells or the monocytic U937 cells. The viability of DAMI and CMK cells, as assayed by trypan blue exclusion, decreased sharply from above 90% to below 50% within 10 days after exposure to HIV-WW. Total cell death in these cultures was observed within 17 days (Fig 1A and B). Cell viability remained above 90% in control CMK and DAMI cultures exposed to an equivalent inoculum of PHA-stimulated, uninfected PBMC supernatant. In contrast, the viability of H9 and U937 cells remained constant and above 90% during the same course of exposure to HIV-WW (Fig 2A and B).

To determine whether the observed cytotoxicity was due to HIV-WW specifically or more generally to exposure to supernatant from HIV-infected PBMCs, we removed virus by filtration or heat-inactivated virus before inoculation. DAMI cells were challenged with an equivalent HIV inoculum depleted of virions by filtration twice through a 0.2- μ m cellulose acetate membrane. In another experiment, CMK or DAMI cells were challenged with HIV-WW after heat-inactivation of virus at 56°C for 30 minutes. Removal by filtration or inactivation by heating of HIV-WW each restored cell viability in the megakaryocytic cell cultures (Fig 3A and B).

HIV infection in CMK or DAMI cells using the laboratory strains HIV-1 IIIB or HIV-2 ROD^{17,36} has not been

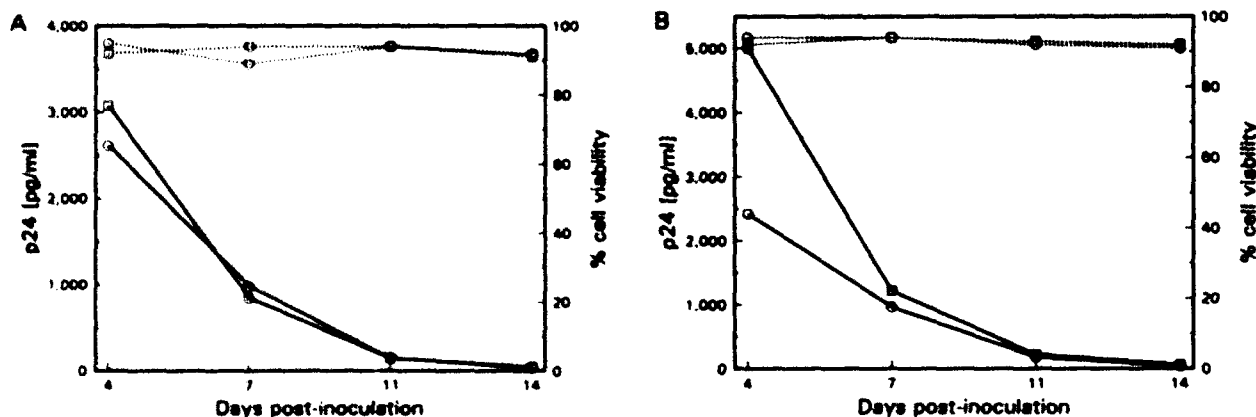


Fig 4. HIV-1 JR-CSF and HIV-RJ9435 are not cytopathic to megakaryocytic cells. CMK or DAMI cells were challenged with 300 μ L (100 ng p24 antigen) HIV-1 JR-CSF or HIV-RJ9435. Supernatant samples were harvested at the indicated times and assayed for viral p24 antigen. Viral p24 antigen levels in CMK (□ — □) and DAMI (○ — ○) cultures are expressed in picograms per milliliter. Supernatant samples from mock-infected cultures were used as negative control. Cell viability in CMK (□ — □) and DAMI (○ — ○) cultures was assessed by trypan blue exclusion. Each value represents the average of two independent measurements. (A) Challenge with HIV-1 JR-CSF. (B) Challenge with HIV-RJ9435.

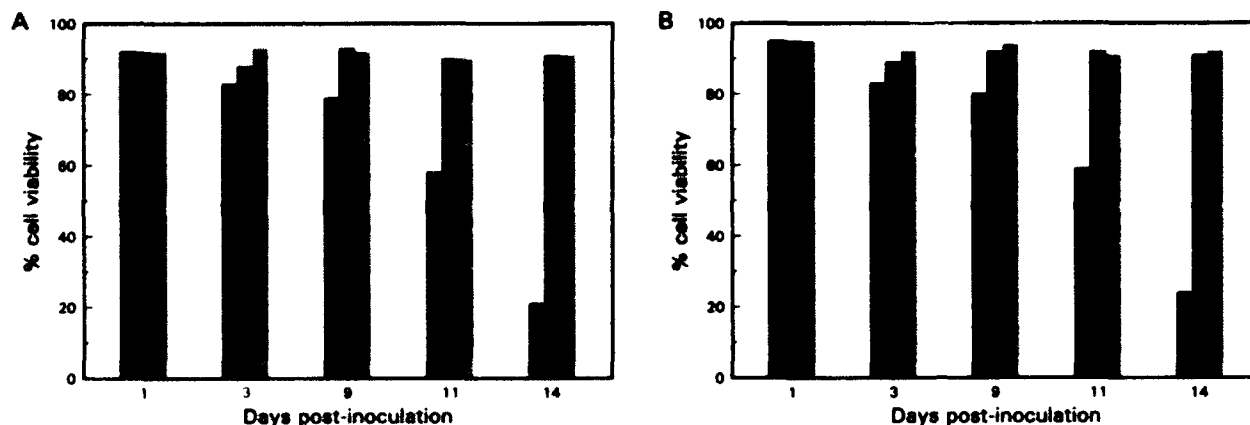


Fig 5. HIV-WW cytopathicity is blocked by neutralizing serum. CMK or DAMI cells were challenged with HIV-WW, as described in Fig 1, in the absence (■) or presence (▨) of neutralizing serum (200 μ L). Control CMK and DAMI cell cultures were mock-infected (■) in the presence of neutralizing serum. Cell viability was assessed by trypan blue exclusion. Each value represents the average of two independent measurements. (A) CMK cell cultures. (B) DAMI cell cultures.

shown to result in any loss of cell viability. Therefore, we further investigated whether the cytopathic effect of HIV-WW on megakaryocytic cells was specific to that isolate or a general property of primary HIV-1 isolates. We challenged CMK and DAMI cells with two primary isolates obtained from nonthrombocytopenic patients. HIV-1 JR-CSF, a PBMC tropic isolate, was isolated from the cerebrospinal fluid of a patient with AIDS dementia^{27,28} whereas HIV-RJ9435 was recovered from an asymptomatic HIV-seropositive individual with neutropenia. Like HIV-WW, these two viruses were amplified by short-term culture in PHA-stimulated PBMCs. CMK, DAMI, H9, and U937 cells were exposed to a 300- μ L inoculum of HIV-1 JR-CSF or HIV-RJ9435 under the same conditions used with HIV-WW. Neither HIV-1 JR-CSF nor HIV-RJ9435 induced cytotoxicity in CMK or DAMI cultures (Fig 4A and B). Cell viability in H9 and U937 cultures remained above 90% (data not shown). Thus, the cytotoxicity to megakaryocytic cells of inocula derived from HIV-WW-infected PBMC cultures

was specific to that virus and not seen with two other primary isolates from nonthrombocytopenic patients. In addition, we found that cytotoxicity was abrogated (Fig 5A and B) when CMK and DAMI cells were challenged with HIV-WW in the presence of a broadly neutralizing serum from an HIV-seropositive donor. This serum was capable of neutralizing under the same conditions both HIV-1 JR-CSF and HIV-RJ9435 infections in PBMCs to 82% and 91%, respectively.

We then investigated whether viral replication played a role in the observed cytotoxicity. CMK or DAMI cells were exposed to HIV-WW in the presence of 250 nmol/L, 500 nmol/L, and 1 μ mol/L 3'-azido-3' deoxythymidine (AZT) to inhibit viral replication. Treatment with AZT did not protect CMK or DAMI cells from the cytopathic effect of HIV-WW. AZT was not toxic to the megakaryocytic cells, as less than 10% cell death was observed in the absence of virus in control cultures treated with AZT at the same concentrations.

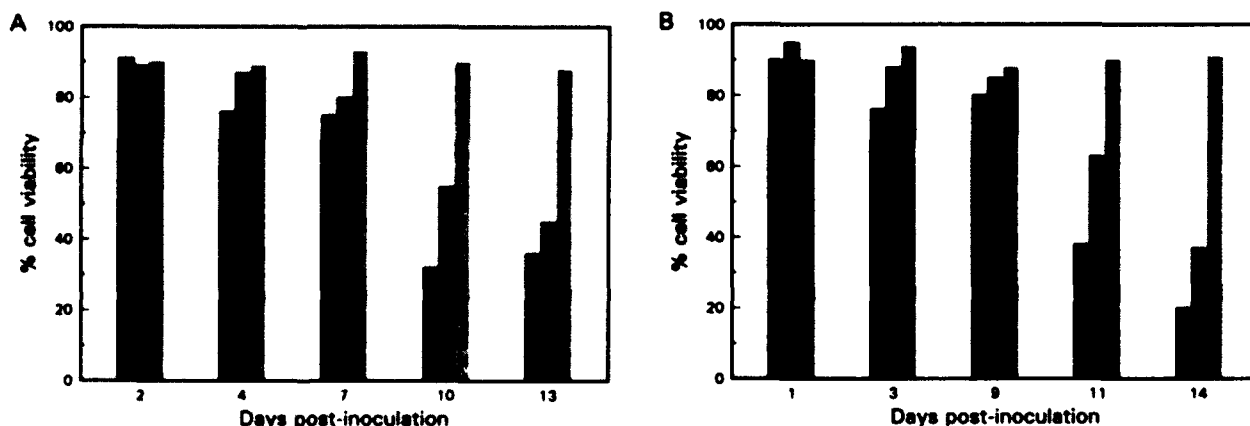


Fig 6. HIV-WW cytopathicity is not blocked by anti-Leu3A. CMK and DAMI cells were challenged with HIV-WW, as described in Fig 1, in the absence (■) or presence (▨) of the CD4-specific monoclonal antibody anti-Leu3A at 5 μ g/mL. Control CMK or DAMI cell cultures were mock-infected (■) in the presence of anti-Leu3A at the same concentration. (A) CMK cell cultures. (B) DAMI cell cultures.

Early events of viral integration and replication were studied by PCR analysis of total cellular DNA isolated at 2, 24, 48, and 72 hours postinoculation. To ensure detection of HIV-WW, despite possible heterogeneity at the molecular level, we selected a very highly conserved region of the HIV genome for amplification, using two gag-specific nested pairs of primers. Although the β -globin gene, used as an internal standard, was detected in equal amount in each of the amplification reactions, we found no HIV-specific sequences in the DNA isolated from CMK, DAMI, H9, or U937 cells. Thus, the megakaryocytic CMK and DAMI cells were not permissive for HIV-WW and the megakaryocyte-specific cytopathicity of the virus was not the result of viral replication. The sensitivity of the PCR assay was such that 1 infected cell in 10^4 cells could be detected. Viral infection, therefore, likely did not occur, or at least occurred below this level of detection and would not explain the observed cytopathic effects. The small amounts of HIV p24 antigen detected early in culture thus represented residual inoculum.

The binding of the viral envelope glycoprotein to the cellular CD4 receptor is a first and critical step in HIV infection. We therefore investigated whether anti-Leu3A, a CD4-specific monoclonal antibody, could inhibit HIV-WW cytotoxicity by blocking the binding of gp 120 to the N-terminus of CD4. Cells were preincubated with antibody (5 μ g/mL) before inoculation with virus. Anti-Leu3A monoclonal antibody did not abrogate HIV-WW-induced cytotoxicity in CMK or DAMI cultures (Fig 6A and B). In contrast, 90% of the cells were viable in control mock-infected CMK cultures with or without anti-Leu3A antibody. These findings indicate that the gp120 epitope that binds to the CD4 N-terminus was not a determinant in HIV-WW cytotoxicity to megakaryocytic cells. Moreover, binding to the CD4 N-terminus was not sufficient in itself to trigger megakaryocytic cell death, because binding with anti-Leu3A antibody to CD4 did not result in cytotoxicity.

DISCUSSION

Impaired megakaryocytopoiesis may be a contributing factor to the thrombocytopenia associated with HIV infection. The presence of the HIV genome in bone marrow megakaryocytes of some seropositive patients has been reported.^{1,8,9} Primary marrow megakaryocytes have a life span of a few days in culture, thus limiting the feasibility of studies of HIV infection in primary cells. Therefore, experiments have been performed in cell lines of megakaryocytic lineage to establish a model of retrovirus-megakaryocyte interactions.^{17,36} However, such studies have been performed to date using laboratory strains of HIV, such as HIV-1 IIIB and HIV-2 ROD. The properties of laboratory strains of HIV may differ significantly from primary isolates, as was recently shown by the failure of soluble CD4 to neutralize primary isolates in vitro at concentrations that effectively blocked infection with laboratory strains of HIV-1.³⁷

We derived an HIV strain from a patient with severe thrombocytopenia and found the virus, HIV-WW, to be highly and specifically cytotoxic to megakaryocytic cells. In contrast, two other primary HIV-1 isolates not associated

with clinical thrombocytopenia were not cytopathic to the megakaryocytic CMK or DAMI cells. Most strains derived directly from HIV-infected patients by short-term culture in PBMCs are not capable of infecting productively permanent cell lines,²⁹ whereas laboratory isolates do replicate in such cell lines. Consistent with this finding, the megakaryocytic CMK and DAMI cell lines were incapable of sustaining HIV-WW replication. PCR amplification detected no HIV viral sequences in these cells after challenge with HIV-WW, with the sensitivity of the PCR assay at 1 infected cell in 10^4 cells. Similarly, treatment with AZT to block replication did not protect the cells from the cytopathic effect of HIV-WW. Despite the absence of replication, the cytopathic properties of HIV-WW were dramatic and specific for cells of megakaryocytic origin and not seen in cells of T-lymphocytic or monocytic origin.

There is considerable heterogeneity in cellular tropism,^{18,22,38,39} cytopathicity,^{21,40,41} and kinetics of replication^{21,23,41} among the various strains of HIV. Although the viral gene products *nef*, *tat*, *vif*, *vpr*, *vpu*, and *vpx* may play an important role in controlling infectivity and viral replication, mounting evidence points to the envelope glycoprotein as a key determinant of cellular tropism and cytopathicity.^{28,29,42-47} Arguably, a low level of HIV envelope components may have been retained in the filtrated viral inoculum and, indeed, we observed that, although cell viability was restored to a great extent, cytopathic effects were not entirely abrogated after filtration. We found, as well, that HIV-WW-induced cytopathicity in megakaryocytic cell lines was not abrogated by anti-Leu3A monoclonal antibody, thereby indicating that the domain of the HIV gp 120 envelope protein that binds to the N-terminus of CD4 was not involved in this phenomenon. Recently, regions of gp120 distinct from the CD4-binding domain have been shown to be critical in determining cell tropism,³⁸ thus suggesting that interactions between several distinct envelope domains may be necessary for viral entry. Identification of the molecular determinants of viral phenotype is essential to an understanding of the pathogenesis of HIV. To this end, we have constructed a genomic library from HIV-WW infected PBMCs to isolate a clone of this novel HIV strain. Studies of its genotype and, in particular, a detailed analysis of the sequence and structure of its envelope glycoprotein should provide insights into the mechanism of its cytopathicity for megakaryocytic cells. Moreover, these studies may show megakaryocyte surface structures distinct from CD4 that specifically interact with components of the viral envelope.

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**IN VITRO EFFECTS OF STEM CELL FACTOR OR INTERLEUKIN-3
ON MYELOSUPPRESSION ASSOCIATED WITH AIDS**

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ABSTRACT

Objective: To determine if the early-acting hematopoietic growth factors stem cell factor (SCF) or interleukin-3 (IL-3) are capable of overcoming the bone marrow suppressive effects of cytokines or drugs involved in the hematologic abnormalities accompanying HIV-1 infection.

Design: In vitro colony formation assays of normal human bone marrow cells exposed to the myelosuppressive medications zidovudine (ZDV), interferon alpha (IFNa), ganciclovir (GAN), or the myelosuppressive cytokines tumor necrosis factor-alpha (TNF) or transforming growth factor-beta (TGF) implicated in HIV dysmyelopoiesis.

Results: SCF (10 ng/ml) was capable of enhancing the numbers of erythroid (BFU-E) colonies in the presence of ZDV or GAN ($p < .05$) and myeloid (CFU-GM) colonies in the presence of GAN or IFN ($p < .05$) relative to controls. IL-3 (10 ng/ml) also improved erythroid colony numbers in the presence of ZDV ($p < .05$) and CFU-GM in the presence of IFN ($p < .05$). Neither factor consistently altered the inhibition of TGF or TNF. The 50% inhibitory concentration (IC_{50}) of the myelosuppressive agents was altered in only one setting: by IL-3 in the presence of ZDV.

Conclusions: These data suggest that SCF or IL-3 may have a therapeutic application in overcoming hematopoietic abnormalities associated with drugs commonly used in the care of AIDS patients. In contrast, they may have less capacity to

overcome the bone marrow inhibitory effects of the endogenous cytokines TNF and TGF.

INTRODUCTION:

Decreased blood cell counts are commonly observed in HIV infected individuals and are the dose limiting toxicity of several important therapeutic agents used in the treatment of AIDS patients. The myelosuppressive drugs ZDV, GAN or IFNa are often administered daily and for prolonged periods (unlike the intermittent use of cancer chemotherapy). Agents which may mitigate the myelosuppressive effects of such drugs should therefore have the capacity to overcome their toxic effects when concurrently present. To that end, we have investigated the hematopoietic stimulatory activity of two growth factors with activity early in the hematopoietic cascade, SCF and IL-3, in the presence of ZDV, GAN or IFNa. We have also studied the effects of SCF or IL-3 on in vitro hematopoiesis in the presence of two cytokines postulated to be myelosuppressive and inappropriately produced in HIV disease, TNF and TGF.^{1,3} These factors would similarly be expected to be present concurrent in with the stimulatory growth factors if the latter were to be used as therapeutics.

Human SCF, also known as c-kit ligand, has a wide range of biological activities most notably within the hematopoietic system.⁴⁻¹⁰ Although alone it has only minimal effects on cell number, SCF in combination with other hematopoietic growth factors is a potent

stimulus for myeloid, erythroid and lymphoid cell proliferation. IL-3 also acts on early bone marrow progenitor cells, but at a later stage of hematopoietic differentiation than SCF. Unlike SCF, it is capable of stimulating the proliferation of blood cells when used as a single agent. The differences between these early acting factors may result in different effects on blood cell progenitors in the presence of myelosuppressive agents and suggest applications for their ultimate use in clinical care.

METHODS:

Cells: Human bone marrow cells were obtained from normal volunteers using protocols approved by the Institutional Review Board of the New England Deaconess Hospital. Light-density mononuclear cells (MNC) were collected following density centrifugation over Ficoll-Hypaque gradients (Pharmacia, Piscataway, NJ). Cells were washed twice in Iscove's Modified Dulbecco's medium (IMDM) (Gibco Laboratories, Grand Island, N.Y.) supplemented with penicillin (250U/ml), streptomycin (250mcg/ml) and 20% fetal calf serum (FCS) (JR Scientific, Inc, Woodland, CA). Bone marrow cells were incubated overnight on tissue culture dishes and nonadherent cells removed and washed in IMDM.

Drugs and Cytokines: Recombinant human SCF (2.3 mg/ml) and recombinant human IL-3 (2.2 mg/ml, $>10^4$ U/mg) were provided by Amgen, Inc. (Thousand Oaks, CA). Zidovudine (Burroughs Wellcome Co, Research Triangle Park, NC) at 20uM was diluted in phosphate buffered saline (PBS). Ganciclovir (Syntex, Palo Alto, CA) was

reconstituted in PBS at 1mg/ml. Recombinant human interferon alpha-2a was kindly provided by Roche Pharmaceuticals (Nutley, NJ) at 10^7 U/ml. Human transforming growth factor beta-1 (R & D Systems, Minneapolis, MN) was diluted according to manufacturer's recommendations and used at 10ng/ml. Recombinant human tumor necrosis factor alpha was kindly provided by Genetics Institute (Cambridge, MA) and diluted to 100ug/ml in PBS. These agents were added to the bone marrow cultures at varying concentrations as indicated below.

Bone marrow colony assays: Light-density adherent bone marrow cells were cultured at a concentration of 5×10^4 cells/ml in 1ml of semisolid methycellulose matrix consisting of 0.9% methylcellulose, IMDM with 30% FCS, 0.8% deionized bovine serum albumin (Sigma, fraction V), 10^{-4} mol/L 2-mercaptoethanol, recombinant human GM-CSF (kindly provided by Genetics Institute) at 1.8nmol/L and recombinant human EPO (kindly provided by AMGEN), at 1U/ml. The cells were cultivated at 37°C and 5% CO₂ for 14 days and colony number scored. Experiments were performed 3-4 times each with each sample performed in duplicate.

Statistical analyses were performed using two-sided student's t test. Differences were considered significant when a $p < .05$ was achieved at all dose levels of inhibitor tested. IC₅₀ was derived by linear regression analysis using mean colony numbers; standard error of IC₅₀ was calculated using data from three independent experiments.

RESULTS:

Addition of SCF to human bone marrow cultures increased the number and size of erythroid (BFU-E), myeloid (CFU-GM) and mixed lineage (CFU-mix) colonies (2.9, 1.3 and 2.7 fold respectively; $p < .05$). In contrast, IL-3 increased CFU-GM (1.5 fold; $p < .05$), but provided no significant additional support of BFU-E or CFU-mix when added in comparable concentrations.

ZDV added to marrow cultures at concentrations similar to clinical serum levels, suppressed BFU-E and CFU-GM in a dose dependent manner (Figure 1). In the presence of SCF, there was significant improvement in the ZDV-induced suppression of erythroid colonies; myeloid colonies increased, but did not achieve statistical significance. The percent suppression of either colony type by ZDV was not statistically significantly different comparing SCF treated cells with no SCF controls and the derived IC_{50} of ZDV was unaffected by the presence of SCF (Table 1).

IL-3 (10 ng/ml) significantly increased BFU-E in the presence of ZDV, but did not affect the number of CFU-GM. The IC_{50} of ZDV was significantly improved by IL-3 ($0.5 \pm 0.1 \mu M$ versus $1.0 \pm 0.2 \mu M$ ZDV; $p < .05$).

GAN at concentrations of 0.1 $\mu g/ml$, 0.2 $\mu g/ml$ or 1 $\mu g/ml$ (concentrations obtained in vivo) suppressed erythroid and myeloid colony numbers in a dose related manner (Figure 1). Compared with controls, SCF (10 ng/ml) was able to significantly increase the numbers of erythroid or myeloid colonies ($p < 0.05$). IL-3 did not induce any significant alteration of either erythroid or myeloid

colony number. GAN IC_{50} was not significantly altered by either SCF or IL-3.

IFNa reduced BFU-E and CFU-GM; SCF and IL-3 were capable of improving CFU-GM colony numbers, but neither significantly altered the numbers of BFU-E nor the IC_{50} for either colony type.

Both TGF and TNF induced a dose related decrease in erythroid or myeloid colonies which neither SCF nor IL-3 affected in a statistically meaningful way.

DISCUSSION:

The studies reported here provide an in vitro assessment as to whether the bone marrow suppression observed in HIV infected individuals, due to either specific drugs or endogenous dysregulated cytokines, may be ameliorated by administration of the growth factors SCF or IL-3. These factors are of particular interest because they act early in the differentiation of hematopoietic cells and may enhance the multiple different cell lineages often suppressed in HIV-1 infection.

SCF was able to increase colony numbers in the presence of myeloid suppressive drugs (ZDV, GAN, IFN), but did not improve colony numbers above control when the suppressive cytokines TNF or TGF were present. Even in the setting in which SCF was capable of increasing colony numbers (ZDV, GAN or IFN) there was no consistent effect on the IC_{50} of the inhibitory factor. These data contrast with Miles et al. who detected a SCF dependent change in the IC_{50} of

ZDV. However, their IC_{50} appeared to vary based on the concentration of erythropoietin in the culture conditions, possibly accounting for the differences in our data.¹¹

IL-3 stimulated increased numbers of CFU-GM under control conditions and in the presence of IFN, but not in the presence of ZDV, GAN, TNF or TGF. The numbers of erythroid colonies were improved in the presence of ZDV and the IC_{50} significantly increased suggesting some protective effect of IL-3 on the toxicity of ZDV. Alterations in the cellular handling of thymidine analogues by IL-3 have been noted by others¹² and may contribute to the effect we observed.

Other than the effect of IL-3 on ZDV suppression of BFU-E, neither IL-3 nor SCF were capable of affecting the IC_{50} and, therefore, the sensitivity of blood cell progenitors to the inhibitory drugs. Rather, it may be that the effect of SCF or IL-3 is simply to improve the overall cell number to an extent that even in the presence of suppressive agents, a significantly higher number of colonies remains.

In the presence of the inhibitory cytokines TNF or TGF, neither SCF nor IL-3 were able to significantly improve the colony numbers. These data suggest mechanisms of myelosuppression by TNF and TGF distinct from those mediating the inhibitory effects of ZDV, GAN or IFN α . TNF and TGF appear to dominantly alter cellular proliferative responses to SCF, IL-3, GM-CSF or EPO rendering the cells less responsive to these stimulatory signals.

The experiments reported here suggest that SCF or IL-3 have

potential therapeutic roles in overcoming the hematopoietic abnormalities associated with certain myelosuppressive drugs commonly used in the care of HIV infected patients. In contrast, these factors may be less able to overcome marrow dysfunction induced by the endogenous inhibitory cytokines, TNF and TGF. The extent to which the inhibitory cytokines play a role in the clinically observed hematopoietic abnormalities of HIV disease remains to be determined and the potential role for these two early acting hematopoietic growth factors in the care of these patients requires further study.

FIGURE LEGENDS

Figure 1: BFU-E (left column) or CFU-GM (right column) in the presence of ZDV (0, 0.05, 0.1 and 1.0uM), GAN (0, 0.1, 0.2 and 1.0uM), IFN (0, 100, 1000 and 5000U/ml), TGF (0, 0.04, 0.4, 2.0 ng/ml) or TNF (0, 0.4, 4.0 and 20 ng/ml) and control, SCF (10 ng/ml) or IL-3 (10 ng/ml). Data presented are the mean and SE.

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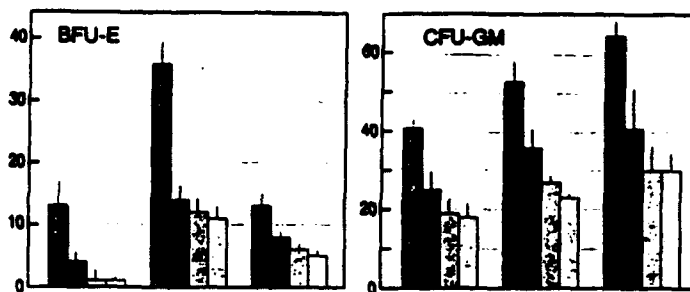
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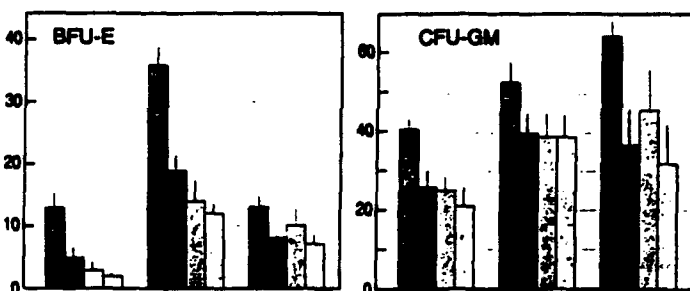
IC50

	ZDV uM	GAN uM	IFN U/ml	TNF ng/ml	TGF ng/ml
BFU CONT	0.5 +/- .1	.55 +/- .1	3500 +/- 1300	10 +/- 1	1.3 +/- .3
BFU SCF	0.8 +/- .2	.8 +/- .1	5000 +/- 500	18 +/- 10	1.3 +/- .8
BFU IL3	*1.0 +/- .2	.9 +/- .6	6000 +/- 1000	16 +/- 3	2.4 +/- .3
GM CONT	1.2 +/- .05	1.2 +/- .6	4000 +/- 400	25 +/- 13	1.5 +/- .4
GM SCF	1.1 +/- .1	1.7 +/- .3	5600 +/- 400	40 +/- 8	2.5 +/- 1.5
GM IL3	1.2 +/- .1	1.2 +/- .4	5200 +/- 400	25 +/- 5	2.5 +/- 1.3

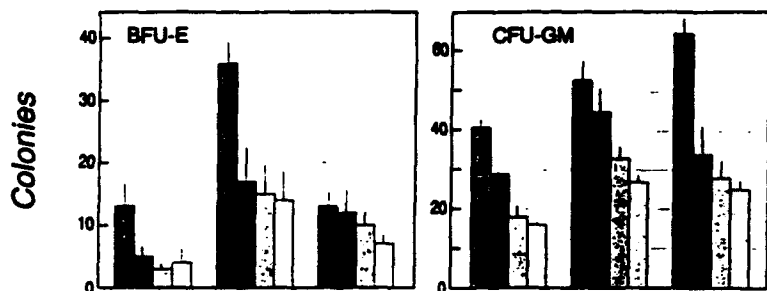
ZIDOVUDINE



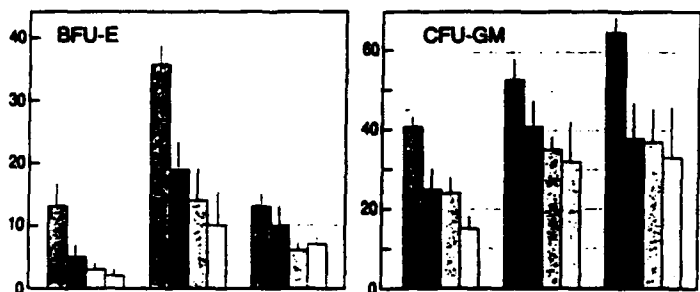
GANCICLOVIR



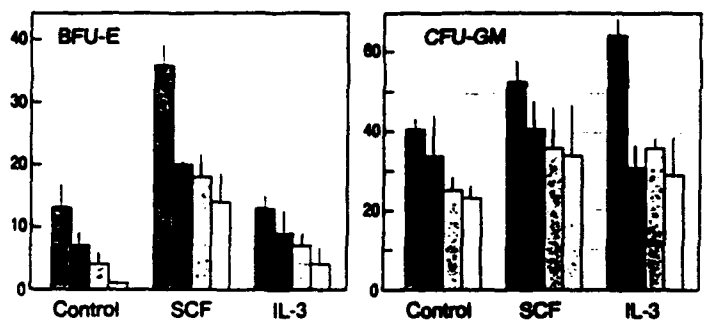
INTERFERON- α



TGF- β



TNF- α



Colonies

Control

SCF

IL-3

Control

SCF

IL-3

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**Stimulation of Human Immunodeficiency Virus Type 1 Expression by
Ceramide**

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transduction

ABSTRACT

Ceramide, an intracellular lipid mediator of tumor necrosis factor alpha (TNF α) action, was studied for its effects on the expression of the proviral Human Immunodeficiency Virus type 1 genome in latently infected myelomonocytic cell lines U-1_{mb} and OM-10.1. Ceramide treatment resulted in a 20 to 100-fold enhancement of HIV production in these cells. Ceramide also enhanced the expression of the chloramphenicol acetyltransferase gene directed by a Human Immunodeficiency Virus type 1 Long Terminal Repeat in transfected U-937 cells, indicating that ceramide acts at the level of viral transcription. These observations suggest that the TNF-ceramide signalling system may be involved in the regulation of HIV expression in certain myeloid cell types.

INTRODUCTION

Ceramide is a recently recognized intracellular lipid second messenger formed from sphingomyelin by the action of a receptor activated sphingomyelinase upon stimulation of various cell types by gamma-interferon, 1,25-dihydroxy-vitamin D₃ or tumor necrosis factor alfa (TNF α) (1-9). The ceramide generated within the cell participates in signals related to cell differentiation or apoptosis (10,11). These effects can be experimentally obtained by the exogenous administration of ceramide (10-13).

Since both TNF α and 1,25-dihydroxy-vitamin D₃ are potent inducers of the expression of the proviral HIV genome, and this induction could involve ceramide as an intermediary (8,9,13-22,24,26), we decided to study the direct effect of exogenous ceramide on the expression of HIV. We examined as models of ceramide modulation of HIV expression two latently infected myelomonocytic cell lines, (U-1_{mb} and OM-10.1). The noninfected counterparts (U-937 and HL-60, respectively) of these lines are relatively well characterized in terms of ceramide signaling (2-5,11,13). The U-1_{mb} and OM-10.1 cell lines produce little or no HIV under basal conditions but significant levels of virus after stimulation (15,21,23,25), and therefore are of value in assessing the role of signal pathways in the induction of viral gene expression.

MATERIALS AND METHODS

Cells

The U-1_{mm} cell line was obtained from the AIDS Research and Reference Reagent Program, National Institute of Health. The OM-10.1 and U-937 cells were obtained from the American Type Culture Collection. The cells were cultured in HEPES buffered RPMI-1640 medium (Gibco) supplemented with 10 % heat inactivated fetal calf serum (HyClone), penicillin, streptomycin, glutamine, sodium pyruvate, MEM nonessential amino acids and glucose.

Chemicals

C-2 ceramide (N-acetylsphingosine, from Biomol Research Laboratories, Inc.), was added to the culture media from a 0.1 M solution in dimethyl-sulfoxide (DMSO) with vigorous agitation, immediately before the addition of the cells. The concentration of DMSO diluent was less than 0.1% in the experiments, was included in appropriate control experiments, and did not interfere with the assays. Recombinant human TNF α was obtained from Genzyme. Initial cell densities in each experiment were adjusted to 5×10^5 cells per ml. Cell counts and viability were determined using a hemocytometer and the Trypan-blue exclusion technique.

Virus quantitation

HIV-1 production was assessed by quantitating core p24 concentration in culture supernatants using the DuPont HIV-1

antigen capture ELISA system according to the manufacturers instructions. Samples were assayed in duplicate. Indirect immunofluorescence staining of methanol fixed cells was done using human HIV positive serum and anti-human IgG-fluorescein-isothiocyanate conjugate as previously described (27).

Transfection of U-937 cells

The pU3R-III CAT plasmid (28) was modified by insertion of the hygromycin-B-phosphotransferase gene, obtained by PCR amplification from the plasmid pREP4 (Invitrogene, San Diego, California) into the single XhoI site (pU3R-III CAT/h) as described in (28). Stable introduction of this expression vector into U-937 cells was accomplished by electroporation. Briefly, 1.5×10^7 cells were washed with PBS, resuspended in 500 μ l PBS, 30 μ g plasmid was added and the cells were electroporated on ice in a disposable electroporation chamber by a Cell Porator instrument (Gibco) at 300 V, 1180 μ F. After ten minutes on ice the cells were grown in RPMI-1640 medium (Cellgro, Mediatech) containing 10 % fetal calf serum, penicillin, streptomycin and glutamine for 24 hours. Selection of stable transfectants was done by culturing the cells for 5 weeks in the above medium supplemented with 400 U/ml Hygromycin-B (Calbiochem).

Chloramphenicol acetyltransferase assays

CAT assays were performed by standard methods (29). Briefly, pU3R-III CAT/h transfected U-937 cells were resuspended in

Hygromycin-free medium at an initial density of 5×10^5 cells/ml and stimulated with various concentrations of ceramide. After five days the cells were harvested, washed with PBS and cell counts and viabilities were determined. Cells were then resuspended at a density of 2×10^7 cells/ml in 250 mM Tris/HCl pH=8.3, frozen and thawed four times and the obtained cellular lysate was centrifuged at 10 000xg at room temperature for ten minutes, incubated for ten minutes at 60 °C and centrifuged as above. CAT assays were done at 37 °C by mixing 0.05 μ Ci α - 14 C-chloramphenicol with cell lysate corresponding to 1×10^6 cells in 170 μ l of 250 mM Tris/HCl pH=8.3 containing 0.5 mM acetyl-coenzyme-A, as in (28,29). After 30 minutes the reaction was terminated by the addition of 500 μ l ethyl-acetate and the acetylated chloramphenicol forms were resolved by thin layer chromatography on IB2 silica plates (J.T.Baker) with chloroform/methanol 95:5 as a developing solution. Plates were then autoradiographed, acetylated and nonacetylated chloramphenicol forms were cut out and counted in a beta liquid scintillation counter. Data presented in this report represent at least three separate experiments.

RESULTS

Consistent with previous studies on non-infected cells (10-12), ceramide induced a dose-dependent inhibition of cell proliferation (Fig. 1 panel B). This was not accompanied by marked cell death during a four day incubation period (Fig.1 panel C), as cell viability remained higher than 85 % throughout the experiment. The concentration dependence of cell growth inhibition observed here is in accordance with previous experiments reported by others on noninfected cells (1). The growth stasis induced by 50 μ M ceramide was accompanied by an approximately 30-fold increase in HIV production by the U-1_{MB} cells (Fig.1 panel A), as judged by the measurement of culture supernatant HIV-1 p24 concentration.

The time course of the effect of ceramide on latently HIV infected cells is shown on Fig. 2. In this experiments the cells, at an initial density of 5×10^5 /ml, were treated with ceramide or DMSO control medium and cultured for 5 days. Cell densities were kept below 2×10^6 throughout the experiment by addition of fresh medium containing ceramide or DMSO diluent. As shown on Fig. 2 panel C, 50 μ M ceramide caused an initial arrest of growth. In parallel, a 20 to 100-fold enhancement of HIV production was seen in both myelomonocytic cell types used (Panel A and B). Consistent with previous reports on the induction of apoptosis by ceramide in HL-60 cells (11), this was accompanied by marked cell death after 4 days in the case of OM-10.1 cells (Panel D). The induction of HIV production by ceramide was accompanied by a significant increase of

the percentage of cells staining positive for HIV antigen expression by immunofluorescence, with increases from 0.01 % to 7-10 % and from 5-15 to 50-60 % for the U-1_{mb} cells and OM-10.1 cells, respectively (Fig. 3). These data demonstrate that ceramide acts by inducing the expression of HIV by latently infected cells, rather than by increasing the production of HIV by a small constitutively expressing subpopulation present in the culture.

To assess if ceramide's effect is due to direct activation of the LTR directed transcription of the HIV provirus or is the result of posttranscriptional effects, we studied the expression of the bacterial chloramphenicol acetyltransferase (CAT) gene directed by an HIV LTR in transfected U-937 cells exposed to ceramide. As shown in Fig. 4, ceramide induced a concentration dependent enhancement of the HIV LTR driven transcription of CAT, as determined by chloramphenicol acetyltransferase activity measurements. This indicates that ceramide acts by enhancing viral transcription. This observation is consistent with previous reports in which ceramide activated NF κ B, an important, though not exclusive activator of HIV transcription. (30).

DISCUSSION

TNF α induces HIV provirus expression in various cell types, including those of the myelomonocytic lineage (15,16,21). It has also been observed that TNF α induces increases in cellular ceramide levels, and that inhibition of proliferation and induction of apoptosis by TNF α can be mimicked by ceramide (8,10,11,26). Thus, it has been postulated, that TNF α action is mediated by ceramide (8,11,26). We have extended these observations and now show that exogenous ceramide activates HIV expression by inducing cells to switch from latent to productive infection. This provides direct support for the model that the HIV inducing effect of TNF α occurs, at least in part, via the ceramide signalling system and involves the stimulation of LTR directed HIV gene transcription.

When the effects of ceramide on HIV expression were compared to that of PMA or TNF α we found ceramide stimulation to be 3-6 fold less potent than the other agents (Results not shown). This could be due to difficulty in cell penetration by ceramide. This hydrophobic molecule may associate primarily with extracellular proteins present in the culture medium (1), may insert into the plasma membrane and fail to equilibrate with the cytosol, or may be metabolized within the cell. Alternatively, the ceramide pathway may be only one of several intracellular signalling pathways triggered by TNF receptor activation. TNF receptor triggering may activate the ceramide and protein kinase-C mediated pathways simultaneously (8), resulting in higher stimulation than that

observed with ceramide alone.

Since increased cellular ceramide levels have been reported during the course of acute HIV infection (22), ceramide signalling may be involved in a positive feedback regulatory system of HIV expression. This system, similar to that described for TNF α (16,21) may represent a potential target for novel pharmacological strategies designed to reduce production of the retrovirus.

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LEGENDS TO FIGURES

Fig. 1.

Concentration dependence of ceramide action.

U-1_{MB} cells at a density of 5×10^5 cells per ml were incubated in the presence of various concentrations of ceramide and viable cell number and supernatant HIV p24 levels were determined at day 4.

Panel A: HIV-1 p24 production.

Panel B: Viable cell counts.

Panel C: Cell viability.

Fig. 2.

Time course of HIV expression by ceramide treated cells.

Panel A: HIV-1 core p24 production by U-1_{MB} cells. : control, : ceramide treated cells.

Panel B: HIV-1 core p24 production by OM-10.1 cells treated with 50 μ M ceramide. : control, : ceramide treated cells.

Panel C: Relative cell growth in the absence or presence of 50 μ M ceramide. and : OM-10.1; and : U-1_{MB} cells. Open symbols represent controls, full symbols represent ceramide treated cells. Initial cell density : 5×10^5 cells/ml.

Panel D: Cell viability in the absence (open symbols) or presence of 50 μ M ceramide.

Fig. 3.

Immunofluorescent staining of control (A) and (C), and ceramide

treated (B) and (D) cells (day 5, 50 μ M ceramide) with HIV positive serum. (A) and (B): U-1_{ms} cells, (C) and (D) : OM-10.1 cells.

Fig. 4.

Enhancement of CAT transcription driven by the HIV LTR by ceramide. U-937 cells transfected by the pU3R-III CAT/h plasmid were exposed to increasing concentrations of ceramide, DMSO vehicle or PMA as a positive control and CAT activities were determined at day 5. Ceramide induced a dose dependent increase in CAT activity.

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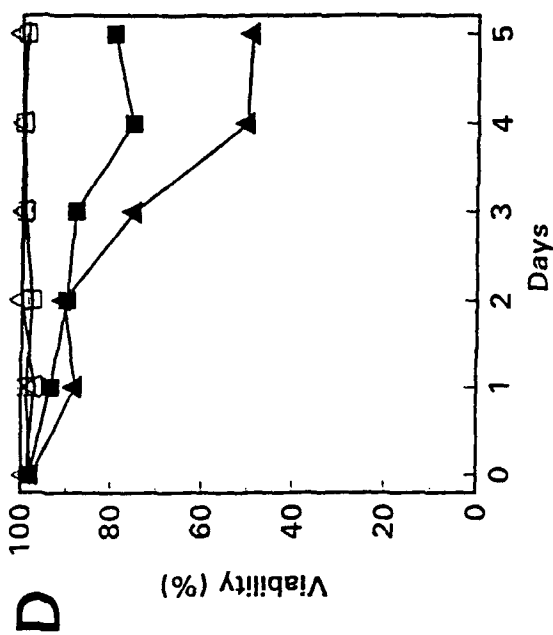
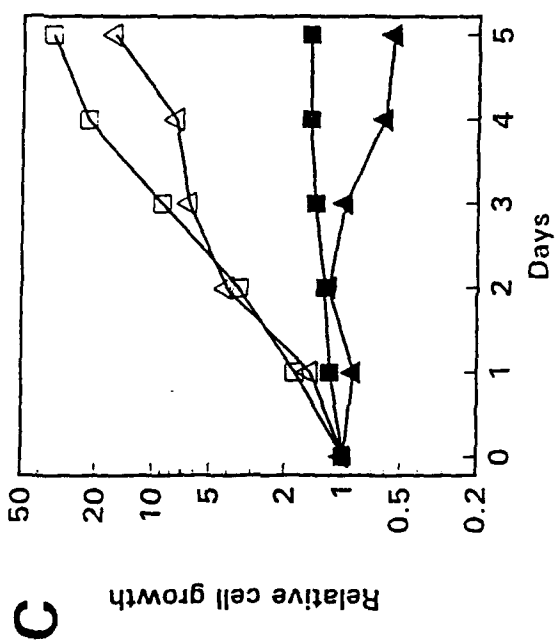
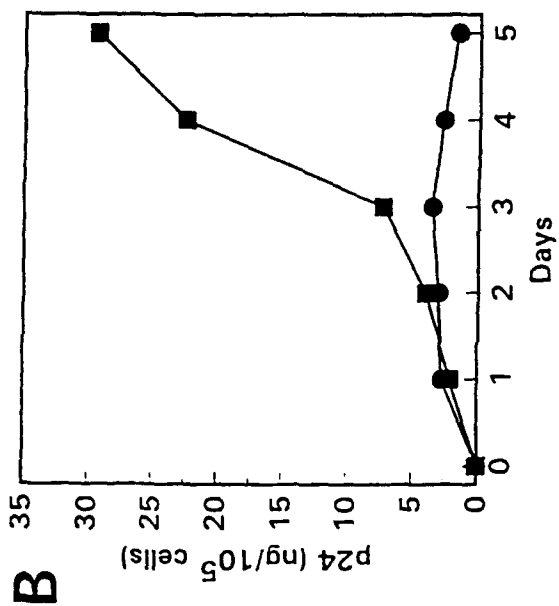
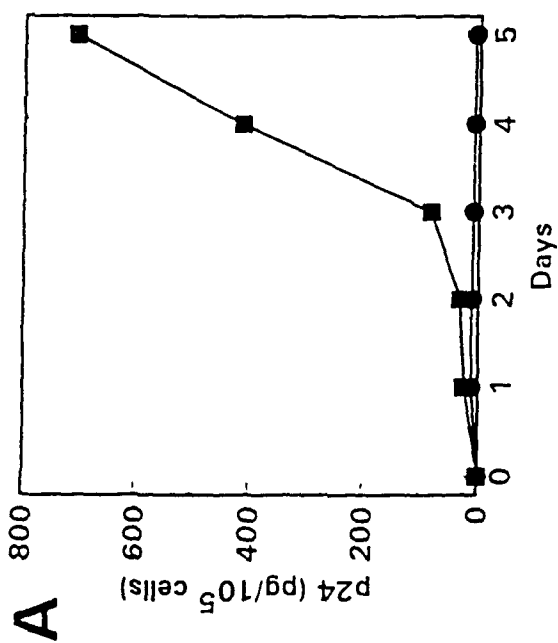
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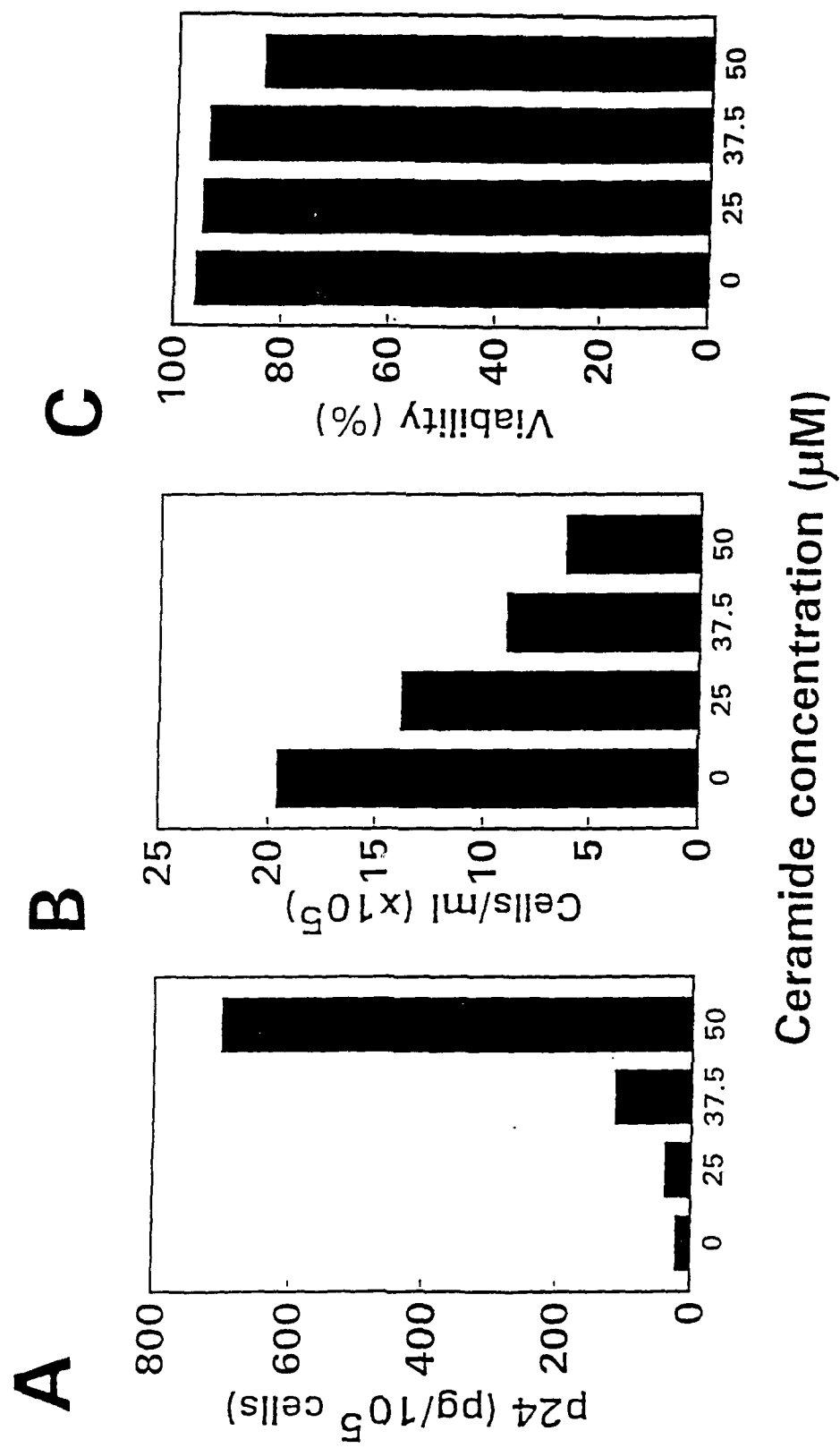
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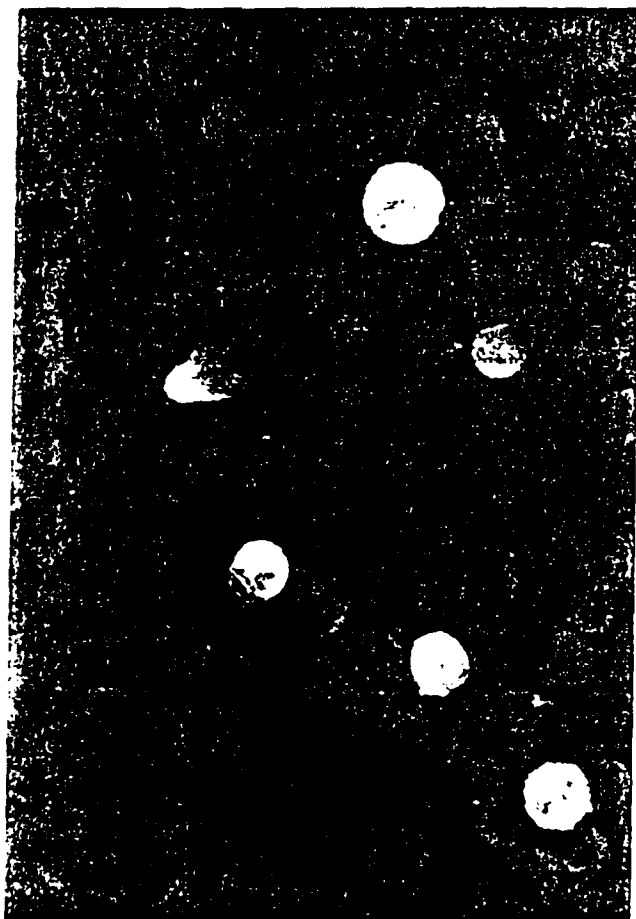
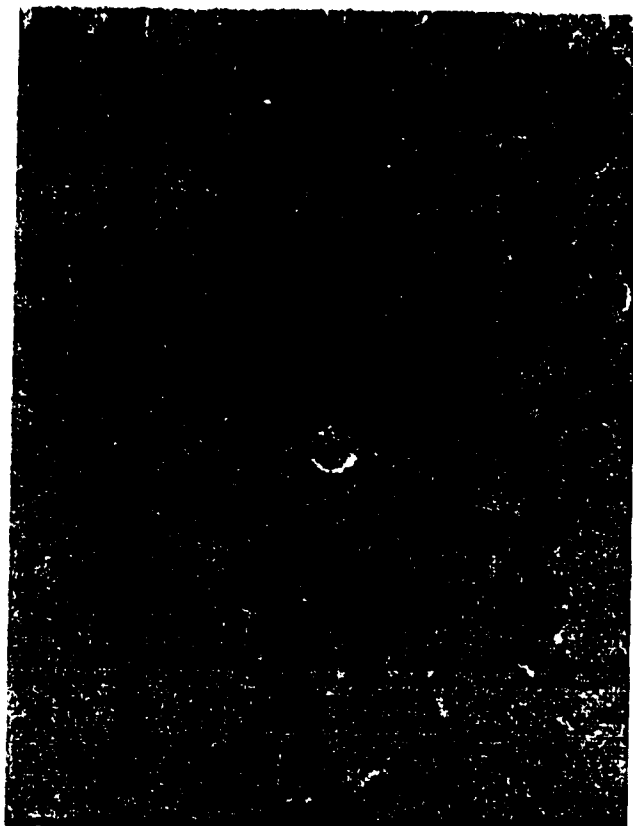
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1.6	2.4	4.8	11.5	0.5	33	% Chloramphenicol conversion
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10

20

30

40

10

20

30

40

DMSO

PMA (10^{-7} M)

μ M ceramide