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Evaluation of multiple parameters of HIV-1 replication cycle in testing of AIDS drugs in vitro

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Summary

Evaluation of the activities of antiretroviral agents and an immunoregulatory compound has been made using two models of HIV-1 infection and three measurements of virus expression. Acute infection of Jurkat cells or chronic/inducible infection in U1.1 cells was monitored at multiple time points after drug treatment. The 50% effective concentrations (EC_{50}) of the HIV-1 inhibitors suramin, 3′-azido-3′-deoxythymidine (AZT), and 2′,3′-dideoxycytidine, as measured by HIV-1 RNA hybridization in Jurkat cells two days after infection, were comparable to EC_{50} values obtained in parallel measurements of extracellular p24 levels and percent HIV-1 IF-positive cells. However, these measurements diverged: at seven days after infection the EC_{50} of AZT was greater than 10 μM when intracellular HIV-1 RNA was assayed, 0.2 μM by IF, and 0.03 μM by p24 assay. Human thymic humoral factor displayed no direct inductive activity in chronic HIV-1 infection in U1.1 cells, while phorbol ester and lymphocyte supernatants induced all parameters. These observations warrant care when interpreting results of only a single assay and suggest that definitive assay of HIV-1 infection requires measurements of multiple parameters of virus expression.

Human immunodeficiency virus; Reverse transcriptase inhibitor; Chronic infection; Multiple parameter evaluation

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Introduction

No single measurement is generally accepted as fully indicative of the course of human immunodeficiency virus type 1 (HIV-1) infection in vitro. Elements of the HIV-1 replication cycle routinely measured to evaluate prospective antiviral drugs include expression of virus antigens or enzymes in p24 (Spector et al., 1989), indirect immunofluorescence (IF) (Hedenskog et al., 1986) or reverse transcriptase assays (Mitsuya et al., 1985), expression of a regulatory protein in Tat-mediated transactivation assays (Felber and Pavlakis, 1988), cytopathic effects in cell viability assays (Mitsuya et al., 1985), and virus transmission in plaque or focus forming assays (Harada et al., 1985). Use of each assay presupposes that some centrality of the HIV-1 replication cycle resides in the parameter examined. The task of drug testing is further complicated by the need for evaluation of different forms of HIV-1 infection, from highly productive and ultimately cytopathic (Barre-Sinoussi et al., 1983; Gallo et al., 1984) to low-productive and/or non-cytopathic (Asjo et al., 1986; Sakai et al., 1988) to those arrested at specific stages of viral expression (Folks et al., 1987; Zack et al., 1990).

In addition to antiretroviral drugs, substances are being evaluated which stimulate or modulate the function of the immune system (Volberding et al., 1983; Reddy et al., 1984). Some of these agents have the potential to affect HIV-1 replication per se, as indicated by in vitro studies with colony stimulating factor in macrophages (Gendelman et al., 1988). Others may affect HIV replication indirectly by inducing secretion of tumor necrosis factor, or by increasing the expression of cellular transcription factors (Rosenberg and Fauci, 1990). Thus, prospective non-antiretroviral AIDS drugs should be evaluated in vitro for their potential to indirectly modulate HIV-1 expression. Inhibition or amplification of steps in HIV replication by anti-viral agents or cytokines also demands quantitation, as well as multiple replica capacity.

We have attempted to address the need for comprehensive AIDS drug testing by comparing three parameters of HIV-1 expression in vitro during acute or chronic/inducible infection at multiple time points after treatment with anti-retroviral drugs or HIV-1 inducers. The parameters selected were the expression of HIV-1 RNA transcripts containing *pol* sequences determined by RNA-RNA solution hybridization (Volsky et al., 1990); extracellular accumulation of core antigen p24, measured by ELISA; and appearance of intracellular bulk HIV-1 antigens as recognized by polyvalent human anti-HIV-1 serum in IF assay. The anti-retroviral drugs used were inhibitors of reverse transcription: 3′-azido-3′-deoxythymidine (AZT) (Mitsuya et al., 1985), 2′,3′-dideoxycytidine (ddC) (Mitsuya and Broder, 1986), and suramin (Balzarini et al., 1986). HIV-1 inducers or modulators used were phorbol 12-myristate 13-acetate (PMA), phytohemagglutinin (PHA) (Folks et al., 1987), thymic humoral factor (THF) (Burstein et al., 1988), or supernatants from untreated or THF-treated lymphocyte cultures. The results of these experiments suggest that proper assay of HIV-1 infection and its response to

drug treatment requires measurement of multiple parameters of virus expression.

Materials and Methods

Test compounds

AZT and ddC were provided by M.-C. Hsu, Hoffmann-LaRoche (Nutley, NJ), suramin (Mobay Chemical Co., NY) was provided by S. Broder, National Cancer Institute, human THF was provided by V. Verhoef (Adria Laboratories, Columbus, OH), and PMA and PHA were obtained from Sigma (St. Louis, MO). The compounds were solubilized as specified by the manufacturers: AZT, ddC and PMA in dimethylsulfoxide and other agents in culture medium.

Cell lines

Subclone U1.1 of U1, a chronically-infected derivative of U-937 cells (Folks et al., 1987), was obtained from J. Laurence (Cornell University, NY). The CD4-positive T lymphoid cell line Jurkat (Schwank and Schneider, 1975) was obtained from W. Greene (Duke University, Durham, NC). CR10 cells, an HIV-1 lysis-resistant subclone of CEM cells, and CEM/N1T-E, CEM cells chronically infected with HIV-1/N1T-E, were derived in this laboratory (Casareale et al., 1987; Sakai et al., 1988). Cells were maintained under standard conditions in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum (FBS) and antibiotics.

Virus preparation and titration

HIV-1/N1T was isolated, characterized, and propagated in chronically infected CR10 cells (CR10/N1T) as previously described (Casareale et al., 1987). Virus production was monitored by measuring the levels of HIV-1 p24 antigen (Coulter, Hialeah, FL) in cell culture supernatants. For concentration of virus, cell-free supernatants from HIV-1 infected cells were sedimented at 17 500 $\times$ *g* for 2 h at 4°C in a JA-10 rotor (Beckman Instruments, Fullerton, CA). The virus pellet was resuspended in fresh culture medium at 1/100 the original volume and stored in aliquots at -80°C. Biological titer (infectivity) of the virus preparation was determined by a three day infection of CEM cells with ten-fold serial dilutions of virus. Viral antigen expression, or $TCID_{50}$ values, were determined by IF assay (Hedenskog et al., 1986). A $TCID_{50}$ unit was defined to be the amount of concentrated virus required to result in 50% IF-positive cells three days after infection; this is approx. 1 pg p24 per cell, depending upon the preparation.

HIV-1 infection

Jurkat cells were incubated for 1 h at 37°C with 2 pg of HIV-1/p24 antigen per cell, or a total of 2×10^9 virus particles as determined by HIV-1 RNA hybridization (Volsky et al., 1990). Cells were then washed with phosphate-buffered saline (pH 7.4), resuspended in culture medium at 2×10^5 cells per ml, supplemented with antiviral drugs at the designated concentration and cultured under standard conditions. At the designated time intervals, aliquots were removed to determine cell viability, p24 concentration in culture supernatants, intracellular HIV-1 RNA, and HIV antigen expression in cells by IF assay. Assays were performed on single samples harvested at designated time points, dose response to each drug was performed three times. Viability was evaluated by Trypan blue exclusion in all treatments including uninfected cells treated with drugs in parallel (not shown). Toxicity greater than 10% was observed in the latter only at the highest concentrations of drugs after seven days of exposure.

Induction of HIV-1 replication in chronically-infected U1.1 cells

U1.1 cells were plated in duplicate at 0.25×10^6 cells per ml in 24-well plates and cultured in the presence of supplements as designated in Table 2. To obtain supernatants of treated peripheral blood lymphocytes (PBL), PBL were isolated by Ficoll-Paque gradient centrifugation from blood drawn from healthy HIV-negative volunteers, resuspended in RPMI-1640 medium containing 10% FBS, and cultured for three days with no further supplements or with the indicated doses of THF. Lymphocytes were pelleted and supernatants were used in U1.1 cultures. At the indicated time points, aliquots were removed to determine cell viability and parameters of HIV-1 expression as described below. The results shown in Table 2 are the average of measurements of duplicate wells.

Measurements of HIV-1 RNA

Cell-associated HIV-1 RNA was measured by liquid hybridization using an HIV-1 RNA assay kit (GeneTrak Systems, Framingham, MA) according to the manufacturer's instructions. Briefly, cells were solubilized at a concentration of 10^7 cells/ml in sample processing buffer containing 5 M GuSCN. A standard curve was constructed using the positive control RNA supplied in the kit and was used to determine amounts of RNA in unknown samples by extrapolation of ^{125}I counts bound. The 'detector probe', pGAP, has been described previously (Pellegrino et al., 1987); it includes sequences of the entire *pol* gene and the 3′ terminus of the *gag* gene. The positive control transcript, pGAPβ, was 3100 nucleotides in length and 1 pg represented approximately 6×10^5 molecules (Thompson et al., 1989).

Assays of HIV-1 antigen expression

HIV-1 p24 capsid protein in culture supernatants from 1–2 × 10^6 cells was measured by the enzyme linked immunosorbent assay (Coulter Immunology, Hialeah, FL) following the manufacturer's instructions. A manufacturer-provided lysis buffer (which includes detergent) was used to dilute samples as appropriate. A standard curve was constructed using standards supplied in the kit and extrapolating from optical density to determine the amount of p24 in unknown samples. IF assays were carried out as previously described (Hedenskog et al., 1986).

Results

Acute HIV-1 infection

The most frequently used in vitro model of HIV-1 infection is primary infection of transformed T lymphocyte lines. We have monitored the course of acute infection of Jurkat cells in dose response to inhibitors of reverse transcription AZT, ddC, and suramin (Fig. 1). The drug concentrations required for 50% and 90% inhibition of virus expression (EC_{50} and EC_{90}, respectively) were extrapolated from data shown in Fig. 1. and are listed in

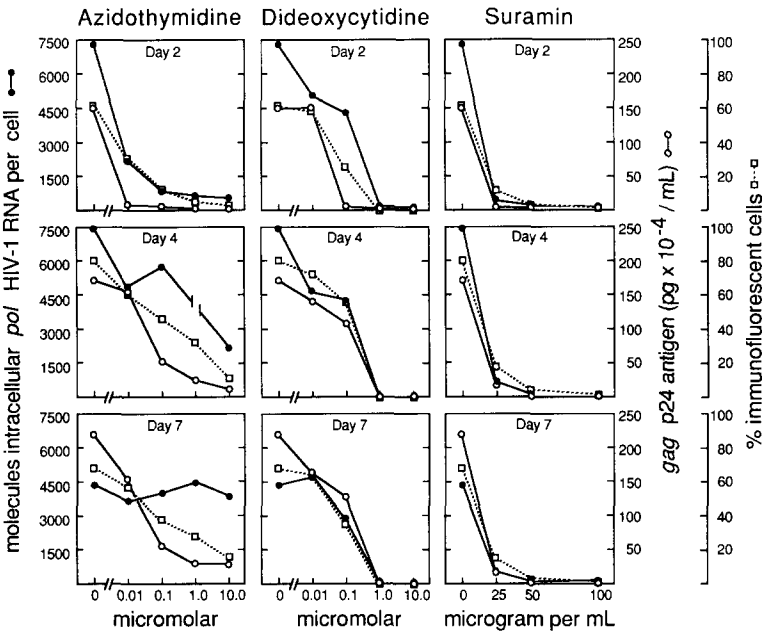

Fig. 1. Inhibition of HIV-1 infection in vitro by AZT, ddC and suramin: comparison of HIV-1 RNA and viral protein detection assays.

TABLE 1

Effects of AZT and ddC on HIV-1 protein and RNA expression

Drug (Days after infection)	EC_{50}[a] (μM)			EC_{90}[b] (μM)		
	IF	p24	RNA	IF	p24	RNA
AZT (2)	0.01	<0.01	<0.01	1.0	<0.01	0.1
AZT (7)	0.2	0.03	>10	>10	>10	>10
ddC (2)	0.08	0.03	0.2	0.5	0.08	0.8
ddC (7)	0.1	0.2	0.2	0.9	0.9	0.9

[a]EC_{50} is the concentration which decreased the IF, p24, or RNA level respectively by 50% compared to that in the control infection. [b]EC_{90} is the concentration which decreased the IF, p24, or RNA level respectively by 90% compared to that in the control infection. Values are extrapolated from the data shown in Fig. 1.

Table 1. Evaluation of the effects of suramin on HIV-1 expression by RNA or either measure of antigens revealed the same sensitivity, an EC_{50} of approx. 12.5 μg/ml. The results of assay for antigen or RNA production seven days after infection of cells treated with ddC were also quite consistent. The dose response to ddC two days after infection was most pronounced in measurement of extracellular p24 yielding an EC_{50} of 0.03 μM and an EC_{90} of 0.08 μM followed by IF with an EC_{50} of 0.08 μM and an EC_{90} of 0.5 μM. However, virus replication continued to be detected by RNA assay, reaching an EC_{50} for ddC at only 0.2 μM. Inhibition of virus expression declined slightly during further exposure to ddC.

The effects of AZT differed dramatically depending upon the infection parameter observed. Like ddC, AZT appeared to be most inhibitory by measurement of viral p24 as compared to viral RNA or IF-positive cells. However, the extent of inhibition by AZT was significantly different from that of ddC. The effects of AZT on virus replication as determined by HIV-1 protein expression, measured either by IF or p24 assay, displayed an EC_{50} of less than 0.01 μM at two days after infection, rising to 0.03 μM for IF and 0.2 μM, respectively, seven days after infection. HIV-1 RNA production increased in the presence of AZT over the course of infection, and reached control (no drug added) levels seven days after infection. RNA expression was directly visualized by autoradiography of the filters containing the RNA-RNA hybrids. As displayed in Fig. 2, although ddC and suramin blocked RNA transcription efficiently, AZT permitted significant RNA production. As noted above, assays of protein production indicated some continued sensitivity to AZT inhibition. Similar results have been obtained upon two repetitions of the same experiment; in particular, the escape from inhibition by AZT was reproducible and consistently revealed first by RNA assay (not shown).

Inducible HIV-1 infection

Insight into HIV-1 replication and its control can also be obtained by examination of the activation of cells which constitutively restrict HIV-1

expression. Such systems are important for drug evaluation since much of the HIV-1 DNA detected in cells in vivo appears to be unexpressed as indicated by the more frequent detection of HIV-1 DNA than RNA in blood cells of HIV-1 infected persons (Schnittman et al., 1989; Psallidopoulos et al., 1990; Hart et al., 1988; Holodniy et al., 1991). This DNA is presumably induced to virus expression upon cell activation (Rosenberg and Fauci, 1990). U1.1 is a chronically infected monocyte line, which produces little HIV-1 until induction by phorbol esters, tumor necrosis factor, or other agents (Folks et al., 1987).

Sample position	Drug	Drug concentration
1, 3, 5 A	AZT	10^{-5} M
1, 3, 5 B	AZT	10^{-6} M
1, 3, 5 C	AZT	10^{-7} M
1, 3, 5 D	AZT	10^{-8} M
1, 3, 5 E	ddC	10^{-5} M
1, 3, 5 F	ddC	10^{-6} M
1, 3, 5 G	ddC	10^{-7} M
1, 3, 5 H	ddC	10^{-8} M
2, 4, 6 A	Suramin	100 μg/ml
2, 4, 6 B	Suramin	50 μg/ml
2, 4, 6 C	Suramin	25 μg/ml
2, 4, 6 D	No drug	ø
2, 4, 6 E	No drug	ø

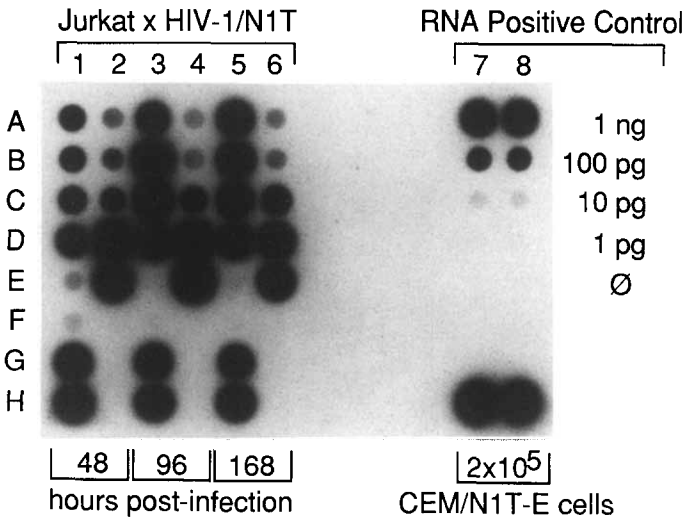

Fig. 2. Detection of HIV-1 RNA by liquid hybridization in HIV-1-infected cells cultured in the presence and absence of antiviral drugs. Jurkat cells were infected with HIV-1 isolate N1T in the presence or absence of drug and harvested at days postinfection as indicated on the autoradiograph figure. Duplicate samples of RNA-positive control standards are in lanes 7,8; where A = 1 ng, B = 1000 pg, C = 10 pg, D = 1 pg, and E = 0 pg; 7, 8 – H are lysates prepared from 2×10^5 CEM cells chronically infected with the N1T-E HIV-1 isolate. Abbreviation: RNA PC, RNA positive control in vitro transcripts. Autoradiography was for 68 hours at −70°C using an intensifying screen.

TABLE 2

Direct and indirect effects of THF on induction of HIV-1 expression in the chronically infected monocyte cell line U1.1

Inducers	Cell viability (No of living cells/ml × 10−6)		HIV-1 p24 antigen in culture supernatants (ng/ml)		HIV-1 RNA (molecules/living cell)		HIV-1-specific immunofluorescence (% positive cells)	
	Day 3	Day 6	Day 3	Day 6	Day 3	Day 6	Day 3	Day 6
Expt. 1								
Added directly to culture medium:								
None	0.82	0.95	4.40	1.25	3	5	0.1	0.1
PHA 5 μg/ml	0.73	1.64	2.50	2.35	24	19	0.4	0.2
PMA 1 μg/ml	0.12	0.50	193.00	205.30	568	1340	62.0	55.0
PMA 0.1 μg/ml	0.19	0.68	190.00	434.10	856	1514	60.0	65.0
THF 0.1 μg/ml	0.94	0.72	1.30	0.95	4	–	0.2	0.1
THF 100 μg/ml	0.92	1.12	1.60	1.25	5	5	0.3	0.1
Added as 20% supernatant from PBL cultured with:								
None	0.61	1.05	76.50	23.40	369	319	28	22
THF 0.1 μg/ml	0.64	1.12	62.00	21.30	270	195	24	17
THF 10 μg/ml	0.64	0.94	110.00	20.00	313	166	30	15
THF 100 μg/ml	0.62	0.95	66.40	18.00	220	123	26	13
Expt. 2								
None	1.34	1.63	1.45	1.3	4	15	N.D.	
Added as 20% supernatant from PBL cultured with:								
None	1.03	0.88	78.3	81.5	356	81	N.D.	
THF 0.1 μg/ml	0.75	0.59	120.0	122.5	435	113	N.D.	
THF 10 μg/ml	0.90	0.72	108.0	69.5	499	100	N.D.	
THF 100 μg/ml	0.52	0.82	102.0	98.2	397	107	N.D.	

We evaluated induction of U1.1 by PMA, PHA, THF, or supernatants from lymphocytes cultured alone or treated with THF for three days. Similar to our analysis of acute infection, we evaluated multiple parameters of HIV-1 expression, here at two time points (Table 2). All parameters tested revealed baseline HIV-1 expression prior to induction. Following induction, the expression of virus differed depending upon the agents and assays. No significant expression was induced by either PHA or THF, however PMA efficiently induced RNA and protein expression as previously reported (Folks et al., 1987; Volsky et al., 1990). To test for indirect effects of THF treatment, PBL were cultivated without mitogens, and with or without THF, and supernatants from these cultures were used in an attempt to activate HIV-1 expression in U1.1. Lymphocyte supernatant was a potent inducer of transient expression of both HIV-1 protein and RNA, however no additional inductive factor was elaborated by lymphocytes cultured with THF. Over the six day culture, the level of induction differed in two trials; HIV-1 RNA expression remained stable in one and declined in the second, while the converse was observed in assay of p24.

Discussion

We have shown discordance between three standard parameters of HIV-1 expression. Unmodulated HIV-1 infection was qualitatively similar when assayed for IF, p24, or intracellular viral RNA; however, the extent of inhibition of HIV-1 replication by antiviral agents differed depending upon the parameter evaluated. New RNA transcription, which is the first detectable indicator of HIV-1 expression (Pellegrino et al., 1991) is also the best indicator of virus escape from inhibition in this work. During the week of treatment, AZT's efficacy decreased from an EC_{50} of <0.01 μM to >10 μM by RNA assay, replication being completely insensitive at 7 days. As indicated by the assay of viral protein, AZT's EC_{50} also rose twenty fold from two days to seven days after infection. Thus in this study, AZT displayed only a transient effect on virus replication. The ability to detect virus expression in the presence of AZT within a week of infection may explain the previous findings of virus expansion upon prolonged cultivation with AZT (Smith et al., 1987). The findings in vitro are consistent with observations of transient clinical efficacy of AZT in AIDS patients (Yarchoan, 1990), however, we can only speculate whether the mechanisms are similar. The generation of AZT-resistant HIV-1 strains as observed in vivo has not been addressed in our in vitro studies (Larder and Kemp, 1989).

As seen during AZT treatment, the virus which is able to replicate during ddC treatment is best detected by RNA assay. In contrast, the three assays revealed the same overall profile of inhibitory activity when infected cells were treated with suramin. The stable inhibition by suramin can be attributed to the multiple effects of this drug on the HIV-1 life cycle; suramin has been shown to

affect both HIV-1 entry (Schols et al., 1990) and reverse transcription (Balzarini et al., 1986). Therapeutic utility of this drug is limited by its toxicity in vivo (Cheson et al., 1987).

The significance of these discrepancies in the evaluation of HIV-1 infection is clear. As a preliminary screen for antiviral activity, assay of protein or RNA expression, or cytopathic effects (Mitsuya et al., 1985) (as is commonly used in high flux surveys) can each be applied with internal reliability. However, upon selection of a given compound for further analysis, virus replication can be monitored accurately only by measurements of distinct products of expression, preferably in different contexts. For example, the parameters evaluated here are independent of the cytopathic effects of HIV-1 and thus can be used for testing replication-competent non-cytopathic HIV strains (Kong et al., 1986; Sakai et al., 1988). Their sensitivity to antiviral agents requires evaluation of measurements different from plaque or focus formation.

Interestingly, in the model of HIV-1 activation in U1.1 cells studied here, the three parameters rose roughly in parallel in response to PMA. This may be attributed to the synchronous response of chronically infected cells to induction of virus expression; in contrast to the multiple phases of virus replication occurring during acute infection (Kim et al., 1989; Pellegrino et al., 1991). The complex mixture of cytokines present in lymphocyte supernatants induced both viral RNA and protein expression in U1.1, although with less coordination than seen with PMA. THF, a member of a family of maturation factors for blood cells considered for combination therapy of AIDS (Reddy et al., 1985), did not elicit the production of any inductive factors in addition to those already synthesized by lymphocytes in short-term culture. Continued exposure of U1.1 cells to lymphocyte supernatants resulted in some reduction in HIV-1 expression; this decline was apparent using assay of either RNA or p24 production. In contrast to previous reports of U1.1 induction (Pomerantz et al., 1990), viability and RNA production remained high over six days of PMA treatment. Such assays will be useful for determining whether immunomodulatory compounds have potential side effects through indirect activation of HIV-1 expression.

In summary, the results reported here suggest that quantitative analysis of HIV-1 replication, particularly when considering modulation of infection, cannot rest on a single existing measurement. Multiple cellular and viral parameters were used for assessing anti-retroviral activity of nucleoside analogues on animal lentiviruses and murine type C retroviruses in vitro (Dahlberg et al., 1987), and in evaluation of HIV-1 inhibition by TIBO derivatives (Pauwels et al., 1990). Such comprehensive approaches to drug evaluation are required to reflect the diversities of the complex HIV-1 replication cycle.

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