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Use of recombinant viruses to assess the pattern of early human immunodeficiency virus breakthrough infection in the presence of stavudine

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A variety of cell lines were infected with replication-defective recombinant retroviruses in the presence of stavudine (d4T). Cells which were infected despite the presence of d4T were isolated and subjected to infection with other retroviruses [replication-competent human immunodeficiency virus (HIV), replication-defective HIV or replication-defective recombinant murine retroviruses]. Each of the host cell types tested had a small subset of cells that were infected with HIV or murine retroviruses in the presence of d4T. Some of these infected cells could be infected repeatedly at high efficiency in the presence of d4T. This phenotype of 'persistent refractoriness' to the antiviral effects of d4T could be overcome by the addition of 5-fluoro-2-deoxyuridine (floxuridine) to d4T. The d4T–floxuridine combination also had potent antiretroviral effects in primary blood mononuclear cells.

Introduction

The measurement of plasma human immunodeficiency virus (HIV) RNA copy number after the initiation of antiviral therapy has provided several insights into the kinetics and dynamics of HIV infection. Initial studies quantitating HIV RNA after the initiation of a non-nucleoside reverse transcriptase inhibitor (NNRTI), nevirapine, indicated a very rapid turnover of plasma HIV (Wei *et al.*, 1995). In those studies there was an initial decline in HIV RNA followed by a rapid increase in plasma viral RNA. These studies with nevirapine demonstrated that the rapid rebound in HIV RNA levels was a consequence of the outgrowth of HIV with phenotypic and genotypic resistance to nevirapine (Wei *et al.*, 1995). Subsequent kinetic analysis indicated a prevalence of nevirapine-resistant HIV-1 variants in pre-treatment plasma of approximately 0·001 of the HIV-1 (Havlir *et al.*, 1996). An *in vitro* model of HIV infection in the presence of a different NNRTI (TIBO) has indicated a very similar prevalence of unselected variants capable of infection in the presence of the drug (Strair *et al.*, 1993)

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Similar clinical and laboratory studies analysing early HIV infection in the presence of 3′-azido-3′-deoxythymidine (AZT) have also been undertaken (Loveday *et al.*, 1995). In contrast to the clinical studies with nevirapine, early HIV infection in the presence of AZT does not appear to be predominated by the outgrowth of AZT-resistant HIV. While the amount of virus circulating in plasma shortly after the initiation of AZT rapidly declines, the remaining circulating virus early after this decline does not contain mutations known to encode resistance to AZT (Loveday *et al.*, 1995). A component of this genetically AZT-sensitive virus may be derived from longer-lived cells with more latent infection (Perelson *et al.*, 1996); however, the amount of virus present and the ultimate emergence of genetically resistant virus containing multiple mutations associated with AZT-resistance implies that there may also be virological, cellular or pharmacological features which allow HIV-1 that is initially genotypically and phenotypically sensitive to AZT to replicate in the presence of AZT. These latter features may impact on the kinetics of virus production in the presence of AZT and allow ongoing replication that supports the development of variants with the multiple mutations known to be associated with high level genetic resistance to AZT.

In prior studies, a quantitative *in vitro* model system has

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been used to analyse a single cycle of recombinant HIV infection in the presence of antiviral drugs (Strair *et al.*, 1993; Medina *et al.*, 1995, 1998). This model system has demonstrated a pattern of virus infection that shares some important characteristics with the pattern of infection seen in the initial clinical studies analysing virus dynamics: (i) early breakthrough infection in the presence of an NNRTI that is dependent on genetic drug resistance; and (ii) early breakthrough infection in the presence of AZT that is, at least partially, independent of the presence of genetic resistance (Strair *et al.*, 1993; Medina *et al.*, 1995). Furthermore, this recombinant virus system has allowed an estimate of the prevalence of unselected NNRTI-resistant variants in a population of unselected replication-competent HIV-1 that has subsequently been approximated by mathematical modelling of HIV-1 kinetics after the initiation of an NNRTI (Havlir *et al.*, 1996; Strair *et al.*, 1993). Therefore, some features of early recombinant virus infection in the presence of antiviral drugs *in vitro* are similar to those described in clinical studies of HIV dynamics after the initiation of an NNRTI (Wei *et al.*, 1995) or AZT (Loveday *et al.*, 1995) and further analyses of this *in vitro* recombinant system may allow a characterization of some of the mechanisms that allow HIV replication in the presence of antiviral drugs.

To determine the predominant mechanisms responsible for early HIV infection in the presence of another nucleoside reverse transcriptase inhibitor, stavudine (d4T), we utilized a similar *in vitro* recombinant system. The results of this analysis indicate that a significant component of early infection in the presence of d4T is a consequence of infection by genetically sensitive virus that has infected a subset of the host cell population which is refractory to the antiviral effects of d4T.

Methods

■ **Cells.** The lymphoid cell lines H9 and JE6.1 were cultured in RPMI 1640 medium supplemented with antibiotics, 2 mM L-glutamine and 10% FBS. Peripheral blood mononuclear cells (PBMC) isolated from healthy HIV-1 seronegative donors were activated with PHA (10 μg/ml) for 72 h prior to infection. After PHA stimulation, PBMCs were maintained in RPMI 1640 supplemented with 10% interleukin-2 (Advanced Biotechnologies), 20% FBS, 2 mM L-glutamine and antibiotics.

■ **Virus.** Production of recombinant HIV–gpt has been described elsewhere (Strair *et al.*, 1993; Page *et al.*, 1990). The amphotropic cell line PA317 was transfected with the recombinant murine retrovirus pLXSN (Miller & Rosman, 1989) and was used as the source of the recombinant MLV–neo virus. Stock preparations of HIV-1 IIIB were harvested from H9 cells by the 'shake off method'. Stock virus infectivity was determined by end-point dilution in MT-2 cells. Virus-induced cytopathic effect was scored 7 days post-infection and the $TCID_{50}$ was calculated with the Reed and Muench equation (Reed & Muench, 1938).

■ **Compounds.** Stavudine (d4T) and floxuridine (5-fluoro-2-deoxyuridine, FUdR) were purchased from Sigma and were dissolved in PBS, sterile-filtered and stored at −20 °C.

■ **HIV-1 RT assay.** HIV-1 production in infected cells was determined by a ^{32}P-based assay as described by Willey *et al.* (1988). RT activity was determined by quantification of ^{32}P bound to DE81 paper by using a Molecular Dynamics phosphorimager. The results are reported as pixel units/μl of the reaction mixture.

■ **Cytotoxicity assay.** A checkerboard analysis of the cytotoxicity of d4T and FUdR alone and in combination was assayed. Triplicate wells of 24-well plates containing 1×10^5 cells were cultured in the absence or presence of various concentrations of each drug alone or in combination. Samples were taken every 2 days for 8–10 days. Drug cytotoxicity was quantified by the MTT reduction assay (Mosman, 1983). The amount of formazan produced in 4 h was determined by dissolving the product in 0·1 M HCl made with 2-propanol and then measuring the absorbance at 570 nm.

Results

Recombinant HIV and murine leukaemia virus (MLV) infection in the presence of d4T

To determine the predominant mechanisms responsible for early HIV infection in the presence of d4T, three different populations of recombinant viruses were utilized (Fig. 1). Recombinant replication-defective HIV–gpt was prepared by transfection of COS cells with complementing plasmids encoding the RNA and proteins necessary for the production of a replication-defective recombinant HIV encoding gpt (Strair *et al.*, 1993; Page *et al.*, 1990) (Fig. 1A). Such viruses are produced without major genetic heterogeneity as the predominant mechanisms responsible for the generation of heterogeneity (e.g. cycles of reverse transcription) are not involved in the production of these viruses. In contrast, replication-defective HIV–gpt made by rescue with replication-competent HIV-1 (Fig. 1B) will contain proteins encoded by the replication-competent virus used for rescue, and are anticipated to have greater heterogeneity. Prior experiments have demonstrated the close relationship between the drug sensitivity phenotype of the recombinant virus and the drug sensitivity phenotype of the virus used to rescue the recombinant virus. For example, the use of an NNRTI-resistant virus to rescue HIV–gpt results in HIV–gpt that is resistant to the NNRTI (Strair *et al.*, 1993; Medina *et al.*, 1998), and the use of an AZT-resistant virus to rescue HIV–gpt results in HIV–gpt that is resistant to AZT (D. J. Medina & R. K. Strair, unpublished). Therefore, recombinant virus produced by rescue with unselected replication-competent virus will be heterogeneous and may reflect the drug sensitivity profile of the virus used for rescue (Strair *et al.*, 1993; Medina *et al.*, 1995). In a prior experiment, the heterogeneity introduced by the replication-competent virus used for rescue resulted in a calculation of the prevalence of HIV genetically resistant to an NNRTI in an unselected population that was very similar to the prevalence subsequently calculated from *in vivo* studies of HIV dynamics after the initiation of an NNRTI (Havlir *et al.*, 1996; Strair *et al.*, 1993).

A comparison of the two virus populations described above (Fig. 1A, B) demonstrated very similar rates of infection

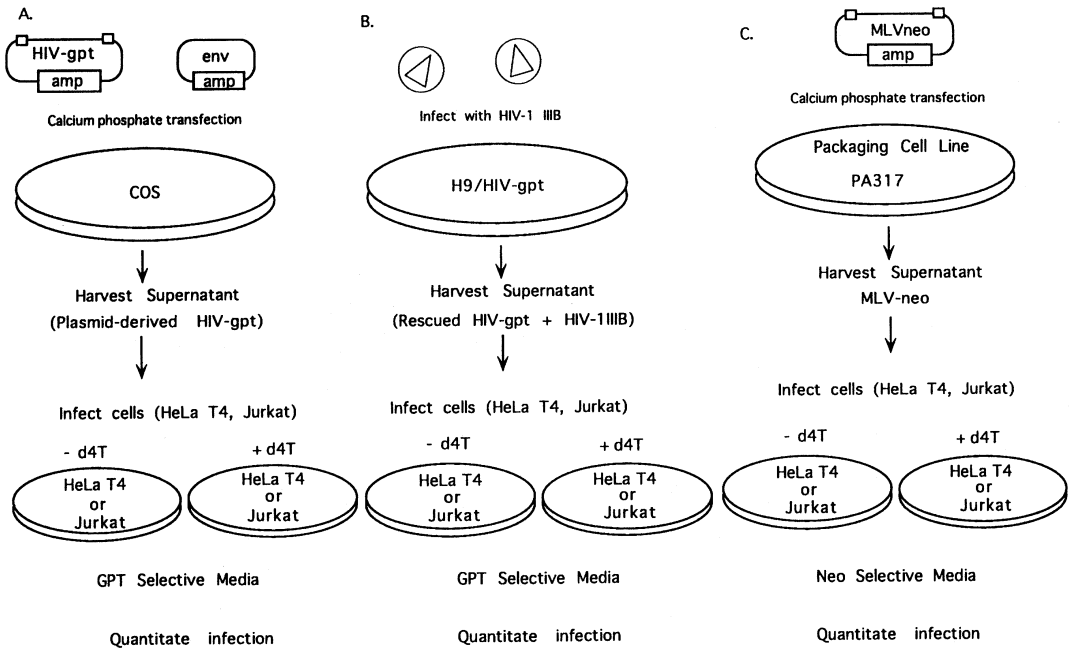

Fig. 1. Schematic representation of the production of the recombinant viruses. (A) HIV–gpt is produced by transfection of plasmids into COS cells. The two plasmids, HIV–gpt and HIV–env (Page *et al.*, 1990) encode the complementing proteins and RNA which allows assembly of the replication-defective recombinant HIV–gpt virus. (B) 'Rescued HIV–gpt' is obtained after infection of the H9/HIV–gpt cell line with HIV-1 IIIB (Strair *et al.*, 1993). The H9/HIV–gpt cell line contains stably integrated HIV–gpt. (C) MLV–neo is obtained after infection of a murine retrovirus packaging cell line, PA317, with a plasmid encoding a replication-defective murine retrovirus containing the *neo* gene.

Table 1. Frequency of HIV–gpt and MLV–neo colony formation in the presence and absence of d4T

Recombinant viruses were used to infect HeLa-T4 cells in the absence or presence of d4T. HIV–gpt was produced by: (i) transfection of COS cells with plasmids (plasmid-derived; Fig. 1A) or (ii) by rescue from the H9/HIV–gpt cell line by HIV-1 IIIB (Fig. 1B). MLV–neo was prepared by transfection of a murine retrovirus packaging cell line, PA317 (Fig. 1C). HeLa-T4 cells were infected with each of the recombinant virus populations in the absence and presence of 50 μM d4T.

Source of virus	No. of colonies		Frequency
	−d4T	+d4T	
Plasmid-derived	$4{\cdot}7 \times 10^4$	80	$1{\cdot}7 \times 10^{-3}$
Rescue with HIV	$3{\cdot}4 \times 10^4$	53	$1{\cdot}6 \times 10^{-3}$
MLV–neo	$9{\cdot}2 \times 10^4$	101	$1{\cdot}1 \times 10^{-3}$

in the presence of 50 μM d4T (Table 1). The similar levels of infection with these two recombinant viruses is in contrast to infections performed in the presence of the NNRTI TIBO (Strair *et al.*, 1993; Medina *et al.*, 1995, 1998). In those prior studies with the NNRTI, there was a 20-fold increased rate of infection with the rescued HIV–gpt virus (Fig. 1B) compared to the virus produced by transfection of COS cells with plasmids (Fig. 1A) (Strair *et al.*, 1993). This disparity of infection rates with the two recombinant virus preparations used for infection in the presence of the NNRTI was interpreted as an indication of the presence of genetic NNRTI-resistant HIV-1 variants. The presence of these variants was subsequently confirmed by genetic analysis of unselected HIV (Najera *et al.*, 1995), and kinetic analysis of the emergence of resistance in treated patients (Havlir *et al.*, 1996; Havlir & Richman, 1994). The absence of a disparity of infection rates with the two virus populations in the presence of either AZT (Medina *et al.*, 1995) or d4T (Table 1) suggests that a component of early infection in the presence of these drugs may be a consequence of infection with virus that is not genetically resistant to the drug.

The virus produced by COS cell transfection of plasmids (Fig. 1A) infected HeLa-T4 cells in the presence of d4T at a relatively high frequency ($1{\cdot}7 \times 10^{-3}$ of the rate of infection in the absence of d4T). To confirm this high rate of infection by recombinant viruses not anticipated to have a high rate of genetic heterogeneity, we used other recombinant viruses and host cells. MLV-based recombinant viruses encoding the *neo* gene (Fig. 1C) showed a similar high rate of infection in the presence of d4T (Table 1). To assure that the high level of infection in the presence of d4T with the viruses depicted in Fig. 1(A, C) was not specific for HeLa-T4 cells, similar infections of Jurkat cells were undertaken. Those experiments

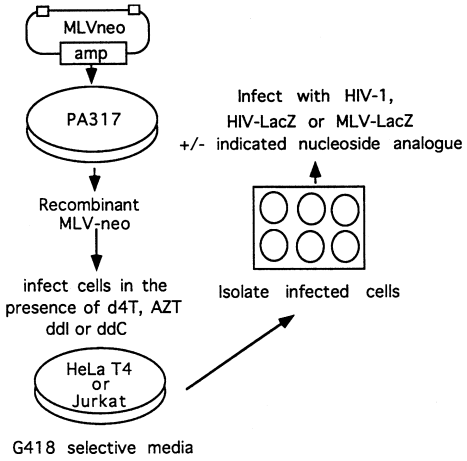

Fig. 2. Isolation and characterization of cells infected with recombinant HIV in the presence of d4T. Recombinant MLV–neo, produced as depicted in Fig. 1 (C), was used to infect cells in the presence of d4T. Infected cells were selected in G418 selective media and individual infected cells were expanded into cell lines. These cells were subsequently infected in the absence and presence of d4T with replication-competent HIV-1 IIIB, recombinant HIV–LacZ (Strair *et al.*, 1993) or recombinant HIV–gpt (Fig. 1 B). Control cells were obtained in an identical fashion with the exception that they were initially obtained after infection with MLV–neo in the absence of d4T.

indicated a similar high frequency of infection of Jurkat cells in the presence of d4T (see Table 3).

The similar rates of infection with virus prepared by plasmid transfection and virus prepared by rescue with replication-competent HIV suggested that features other than genetic resistance might be contributing to early *in vitro* HIV breakthrough in the presence of d4T. This interpretation was supported by evidence of high rates of infection with MLV-based recombinant virions (also anticipated to have a low level of genetic heterogeneity). Infections of Jurkat cells indicated that the high rate of infection in the presence of high concentrations of d4T was not cell line specific. Although it is possible that a low frequency of genetically resistant virus present in the replication-defective recombinant HIV obtained by COS cell transfection and/or MLV virus populations was solely responsible for the infections seen in the presence of d4T, the similar rates of infection seen with both viruses and the lack of an increase in infection with virus obtained by rescue with replication-competent virus indicated that a component of early HIV breakthrough infection in the presence of d4T was likely to be unrelated to infection by genetically resistant virus.

Isolation and characterization of cells infected in the presence of d4T

The presence of a selectable marker gene in the recombinant retroviruses depicted in Fig. 1 allowed the direct isolation of cells infected by these viruses in the presence of d4T (Fig. 2) and the demonstration that a component of the infection was a consequence of infection by viruses that were not genetically

Table 2. Colony formation and persistent resistance after HeLa-T4 infection with MLV–neo virus in the presence and absence of various antiretroviral agents

Individual HeLa-T4 cells infected with MLV–neo (produced as depicted in Fig. 1C) in the presence of the indicated nucleoside analogue were selected in G418 selective media and expanded into cell lines. These cells were then assayed for infection by a recombinant MLV–LacZ (Strair *et al.*, 1991) in the absence and presence of the same nucleoside analogue. Forty-eight hours after infection, the cells were stained for β-galactosidase activity. If the level of infection (number of colonies) in the presence of the nucleoside analogue was $\geqslant 50\%$ of the level of infection in the absence of the nucleoside analogue, the cells were said to be 'persistently refractory' to the antiviral effects of the nucleoside analogue. No control cell lines (HeLa-T4 selected in G418 selective media after infection with MLV–neo in the absence of the nucleoside analogue) demonstrated 'refractoriness' to the antiviral effects of the nucleoside analogue. The concentrations of the nucleoside analogues used were as follows: d4T, 50 μM; AZT, 10 μM; ddI, 50 μM; ddC, 10 μM.

Drug	No. of colonies	No. of persistently resistant colonies	Frequency
d4T	63	23/63	$2{\cdot}5 \times 10^{-3}$
AZT	12	2/12	$2{\cdot}2 \times 10^{-4}$
ddI	19	4/19	$4{\cdot}4 \times 10^{-4}$
ddC	17	2/17	$2{\cdot}2 \times 10^{-4}$
Control	9050	0/15	0

resistant to d4T. HeLa-T4 cells infected with MLV–neo (Fig. 1C) in the presence of d4T were isolated, cloned and expanded into cell lines that were subsequently infected with recombinant HIV, recombinant MLV (MLV–LacZ; see Strair *et al.*, 1991, 1993) or replication-competent HIV-1. As demonstrated in Table 2, approximately 37% of the isolated HeLa-T4 cells were repeatedly refractory to the antiviral effects of d4T (they could readily be infected with recombinant MLV–LacZ in the presence of 50 μM or 100 μM d4T). The same cells readily infected with recombinant MLV in the presence of d4T were also readily infected with recombinant HIV or replication-competent HIV-1 in the presence of d4T (data not shown). Similar experiments with AZT, ddI and ddC indicate that refractoriness to the antiviral effects of each of these nucleoside analogues contributes to early breakthrough infection *in vitro* in the presence of the drug (Table 2). A comparison of the prevalence of cells refractory and persistently refractory to the antiviral effects of these drugs is shown in Table 2. The prevalence of cells persistently refractory to the antiviral effects of d4T is higher than the prevalence of cells persistently refractory to the antiviral effects of AZT, ddI or ddC. These studies directly demonstrate a subpopulation of host cells that are infected with recombinant viruses in the presence of d4T and can be infected repeatedly with other recombinant viruses

Table 3. Infection of Jurkat cell clones sensitive and resistant to d4T

JE6.1 cells infected with MLV–neo (Fig. 1C) were selected in G418 selective media and expanded into cell lines. Bulk refers to an unselected population of JE6.1 that were initially infected with MLV–neo in the presence of 50 μM d4T. R clones represent individual clones of cells initially infected with MLV–neo in the presence of 50 μM d4T. C clones represent individual clones initially infected with MLV–neo in the absence of d4T. JE6.1 is the parental cell line. These cells were infected with MLV–LacZ in the absence and presence of 50 μM and 100 μM d4T. Forty-eight hours after infection with MLV–LacZ, the cells were stained for *β*-galactosidase activity (Strair *et al.*, 1991). The data represent the percentage of cells staining for *β*-galactosidase after infection in the presence of d4T in comparison to the percentage of cells staining for *β*-galactosidase after infection in the absence of d4T, i.e. (% of cells staining for *β*-galactosidase after infection in the presence of d4T)/(% of cells staining for *β*-galactosidase after infection in the absence of d4T) × 100. −, < 20%; +, 20–40%; ++, 41–60%; +++, 61–80%; ++++, > 81%.

Clone	No Drug	50 μM d4T	100 μM d4T
Bulk	++++	++	+
R1	++++	++++	++++
R3	++++	+++	++
R4	++++	−	−
R7	++++	−	−
R12	++++	++++	++++
R14	++++	+	−
R15	++++	++++	++++
R16	++++	+	−
R19	++++	−	−
R20	++++	++++	+++
R21	++++	++++	++++
R22	++++	++	+
R26	++++	+	−
R29	++++	−	−
R33	++++	+++	+
R36	++++	++++	++
C2	++++	−	−
C23	++++	−	−
C26	++++	−	−
C27	++++	−	−
JE6.1	++++	−	−

or replication-competent HIV in the presence of d4T. These cells were not pre-selected by exposure to antiviral drugs.

A high percentage of Jurkat cells (JE6.1) infected in the presence of d4T also were persistently refractory to the antiviral effects of d4T (Table 3). Infections with replication-competent HIV-1 demonstrated that the refractoriness to infection detected in these clones was not a phenomenon solely associated with recombinant viruses (Fig. 3). The d4T IC_{50} of these infections with replication-competent HIV was 50–350-fold higher than the d4T IC_{50} determined by infection of parental JE6.1 cells (Table 4). This marked increase in d4T IC_{50} is in contrast to the only moderate increase in d4T IC_{50}

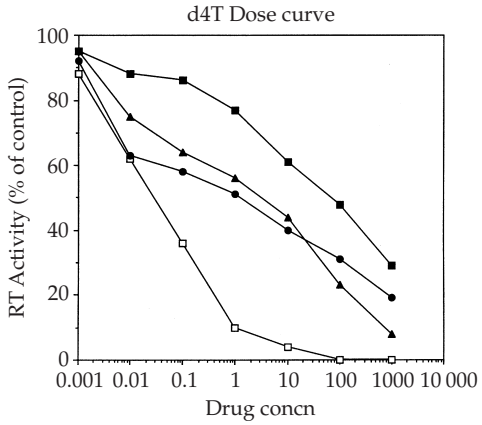

Fig. 3. Infection of JE6.1 cell clones refractory to the antiviral effects of d4T [D4T bulk (▲) is a mixed population of cells initially infected with MLV–neo in the presence of d4T; D4T R1 (■) and D4T R3 (●) are isolated clones of cells initially infected with MLV–neo in the presence of d4T and subsequently demonstrated to be persistently refractory to the antiviral effects of d4T] and a control clone of JE6.1 cells (□) with HIV-1 IIIB in the presence of various concentrations of d4T. RT activity was assayed and compared with that for a control infection in the absence of d4T.

seen when d4T-resistant virus selected *in vitro* or *in vivo* is used to infect unselected cells (Lacey & Larder, 1994; Lin *et al.*, 1994). Therefore, the relative resistance to d4T in the infection of these refractory clones is far greater than the degree of resistance seen with virus that has been selected for prolonged periods either *in vitro* or *in vivo*.

A subset of the persistently refractory cells (approximately 20%) were also refractory to the antiviral effects of AZT. Cells refractory to the antiviral effects of d4T and AZT were not refractory to ddI, ddC or TIBO (data not shown).

These results provide support to the concept that a component of early infection of these cell lines *in vitro* occurs as a consequence of mechanisms independent of genetic drug resistance. The phenomenon of early virus breakthrough in the presence of these nucleoside analogues has been demonstrated with replication-defective recombinant HIV, replication-competent HIV and two replication-defective recombinant murine retroviruses. In addition, early infection as a consequence of cellular features has been seen with two different host cell types (HeLa-T4 and Jurkat 6.1).

As has been demonstrated previously for AZT (Medina *et al.*, 1996), a component of the refractoriness to the antiviral effects of d4T can be reversed by the addition of FUdR (Table 4). The antiviral efficacy of the combination therapy, as measured by the d4T IC_{50}, is markedly improved with combination therapy. Prior studies of the antiviral efficacy of FUdR have demonstrated limited antiviral efficacy of FUdR alone, but marked antiviral efficacy of combined AZT and FUdR (Medina *et al.*, 1996). Table 4 shows the capacity of the FUdR–d4T combination to reverse some of the cellular refractoriness to d4T described above. The FUdR–d4T combination has marked antiviral activity in cells demonstrated to

Table 4. d4T/FUdR susceptibility in cells sensitive and refractory to the antiretroviral activity of d4T

Jurkat (JE6.1) cells that were refractory to the antiviral activity of d4T (a bulk population of cells, D4T bulk, and two cell lines derived from individual cells, D4T R1 and D4T R3) and control JE6.1 cells were infected with HIV-1 IIIB in the presence of 0·001 μM d4T, 0·01 μM d4T, 0·1 μM d4T, 1 μM d4T, 10 μM d4T, 100 μM d4T and 1000 μM d4T in the presence of 0·005 μM FUdR, 0·01 μM FUdR or 0·025 μM FUdR. The IC_{50} represents the concentration of d4T required for 50% inhibition of RT activity on day 6 of infection. The highest concentration of FUdR (0·025 μM) in the absence of d4T resulted in a 17% reduction in HIV RT activity on day 6.

	JE6.1		D4T bulk		D4T R1		D4T R3	
Treatment	IC_{50} (μM)	Sensitivity fold	IC_{50} (μM)	Sensitivity fold	IC_{50} (μM)	Sensitivity fold	IC_{50} (μM)	Sensitivity fold
d4T	0·02	3	74	2				
d4T + 0·005F	0·003	7	0·8	4	0·9	82	0·1	20
d4T + 0·01F	0·001	20	0·04	75	0·2	370	0·03	67
d4T + 0·025F	0·0008	25	0·009	333	0·01	7400	0·007	286

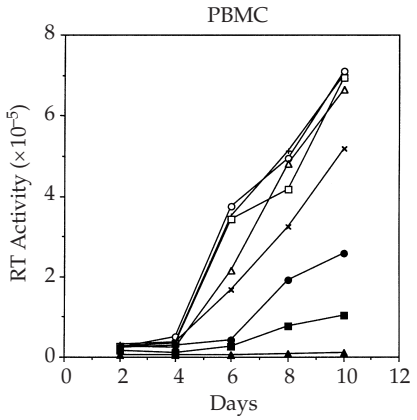

Fig. 4. A d4T–FUdR combination inhibits HIV-1 infection of PBMCs. PBMCs were infected with HIV-1 in the absence of drug (+), with d4T alone (0·01 μM; ×), with various concentrations of FUdR alone (○, 0·005 μM FUdR; □, 0·01 μM FUdR; △, 0·025 μM FUdR) or with combinations of FUdR and 0·01 μM d4T (●, d4T + 0·005 μM FUdR; ■, d4T + 0·01 μM FUdR; ▲, d4T + 0·025 μM FUdR).

be persistently refractory to the antiviral effects of d4T. To assure that FUdR did not inhibit the antiviral activity of d4T in normal unmanipulated PBMC, HIV-1 was used to infect PBMC in the presence of various concentrations of d4T and FUdR. As demonstrated in Fig. 4, the d4T–FUdR combination resulted in markedly enhanced antiviral activity (compared to d4T alone) in PBMC. FUdR alone resulted in minimal antiviral activity.

Discussion

Recent clinical analyses have emphasized the potential of prolonged suppression of HIV viraemia when antiviral drug combinations are used (reviewed in Finzi & Siciliano *et al.*, 1998; Gulick *et al.*, 1997). However, long term eradication of virus has not yet been consistently demonstrated and virus regrowth with cessation of antiviral drugs can occur. In the face of this uncertainty, more detailed information concerning the mechanisms contributing to HIV breakthrough in the presence of antiviral drugs is needed. Although the clinical relevance of the studies presented in this report are unclear, the heterogeneity of cell types hosting *in vivo* HIV infection raises the possibility that similar 'cellular' mechanisms contribute to HIV breakthrough at some point in the natural progression of the disease.

In this report we utilized an *in vitro* model of HIV infection to provide two lines of evidence that early HIV breakthrough infection in the presence of d4T is not solely a consequence of infection by HIV with genetic drug resistance. Initial studies demonstrated that the frequency of HIV infection in the presence of d4T was very similar with several stocks of virus predicted to have significant differences in genetic heterogeneity. These studies suggested that any pre-existing unselected d4T-resistant HIV in the population of HIV used to rescue the replication-defective HIV was not detected above the very high level of infection occurring with the other virus populations. The fact that nearly 50% of the Jurkat cells infected with HIV in the presence of d4T are readily re-infected in the presence of high concentrations of d4T provides definitive evidence of a high level of infection in the absence of genetic d4T resistance. These results are not limited to recombinant HIV and were also demonstrated with MLV-based viruses as well as replication-competent HIV.

A subset of the cells infected with HIV in the presence of d4T does not have a persistent phenotype of being refractory to d4T. These cells may have been infected as a consequence of cell cycle related phenomena, intravirion reverse transcription (Zhang *et al.*, 1994) or other viral, cellular or pharmacological features that are not characterized by persistent cellular phenotypic change.

Previous studies have demonstrated that the combination of FUdR with AZT or d4T has significant antiretroviral activity (Medina *et al.*, 1996; Ahluwalia *et al.*, 1996). Furthermore, the combination of FUdR and AZT has potent antiretroviral

activity in cells refractory to the antiretroviral activity of AZT (Medina *et al*., 1996). These studies demonstrate the capacity to improve the antiviral efficacy of d4T by the addition of drugs such as FUdR with the capacity of interacting with the biochemical mechanisms responsible for AZT and/or d4T metabolic activation. This increased antiviral efficacy in cells refractory to the antiretroviral effects of AZT or d4T may have therapeutic implications for the treatment of early HIV infection in the presence of d4T (e.g. cells infected with d4T-sensitive HIV in the presence of d4T). As this early replication may contribute to the ultimate emergence of genetic resistance, determination of the mechanisms contributing to the presence of virus after initiation of therapy may impact on efforts to achieve long term suppression of the virus.

In summary, we have demonstrated that early HIV breakthrough infection *in vitro* in the presence of d4T is not solely a consequence of infection with virus that is genetically resistant to d4T. As with AZT, infection with drug-sensitive virus is a major mechanism of infection early after the introduction of the drug *in vitro*. Further analyses of the mechanisms responsible for HIV breakthrough in the presence of antiviral drugs are essential to efforts to define drug combinations that provide durable suppression of HIV infection and viraemia.

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