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Generation of multiple drug resistance by sequential in vitro passage of the human immunodeficiency virus type 1

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Summary. We have sequentially passaged both laboratory and clinical isolates of the human immunodeficiency virus type 1 (HIV-1) in MT-4 cells in the presence of increasing concentrations of different drugs to derive viral variants that are multiply resistant to various combinations of ddC, ddI, d4T and AZT. The EC_{50} values obtained for the viruses thus generated varied between 50–100 times above those of parental wild-type strains in the case of AZT, 20–30 times for d4T, but only 10–15 times for ddI and ddC. Cultivation of AZT-resistant viruses in the presence of increasing concentrations of ddI yielded viruses that were resistant to the latter compound, with no apparent decrease in susceptibility to AZT. Sometimes, viruses selected for resistance against ddI were cross-resistant as well against ddC, although most viruses selected for resistance to ddC were not cross-resistant to ddI. Combinations of two or three of these compounds inhibited replication of HIV variants that displayed resistance to the same drugs when tested individually. No emergence of drug resistance was demonstrable when combinations of drugs were employed simultaneously in these selection protocols or when single drugs were used in concert with interferon-2α or high dilutions of virus-neutralizing antisera. Cloning and sequencing of some viruses resistant to each of AZT, ddI, and ddC revealed the simultaneous presence of mutations at sites 41, 74, 184 and 215 within the HIV pol gene open reading frame.

Introduction

The use of 3′-azido-3′-deoxythymidine to treat HIV-1 infected individuals has resulted in both improved survival and quality of life [4, 24, 25]. The use of anti-HIV nucleosides has furthermore been shown to both stimulate numbers of CD4 positive lymphocytes and to cause diminutions in levels of circulating viral p24 antigen [9, 25]. Nonetheless, prolonged therapy with AZT and other drugs has commonly resulted in ultimate treatment failure. Reasons for

progression of HIV-associated disease in the face of antiviral therapy include the fact that nucleoside compounds act by antagonizing the HIV reverse transcriptase and not by affecting postintegrational events in the viral life cycle. Thus, continued viral production by cells that already harbour integrated proviral DNA in their nuclei can occur, resulting in each of direct virus-induced cytopathicity, production of potentially immunosuppressive viral proteins, involvement of viral proteins in antibody-dependent cellular cytoxicity reactions, and induction of cytokines that may, under some circumstances, cause further immunosuppression through direct or indirect mechanisms.

In addition, a decline in specific immune responsiveness against HIV-associated antigens could give rise to higher levels of overall viral replication. The correspondingly higher number of reverse transcription events, now involved in infection of new cells, could give rise to mutations in the viral polymerase, with the potential to encode HIV drug resistance. Thus, it is not surprising that important correlations between diminutions in CD4 counts and development of viral drug resistance have been reported by several groups [1, 16, 26].

An alternative explanation, however, is that HIV drug resistance may develop under conditions of selective pressure exerted by the very drugs used in antiviral chemotherapy. This possibility must be considered seriously, in view of the error-prone nature of the viral RT and the likelihood that mutations with the potential to encode viral drug resistance constantly occur. Previous research has shown that viruses which possess such mutations may be selected in tissue culture over multiple cycles of in vitro growth in increasing concentrations of drug from viruses that were originally wild-type in nature [5, 13]. HIV-1 resistance against non-nucleoside inhibitors of reverse transcriptase can be quickly selected under similar conditions [15, 17].

Combinations of various antiviral nucleosides have been shown to act synergistically in tissue culture to antagonize HIV-1 replication [3, 10, 11]. Such synergy has also been reported with regard to drug-resistant variants of HIV-1 [6, 19]. It is important to assess whether multiple resistance against different compounds may emerge under conditions of antiviral chemotherapy. We now report the selection in vitro of HIV-1 variants that are multiply resistant to each of AZT, ddI, and ddC. In each instance, resistance was first generated using increasing concentrations of a single nucleoside; viruses thus generated were subsequently replicated in increasing concentrations of other drugs. Multiple drug resistance generally occurred without any loss in resistance capacity for the compound used in the initial selection procedure.

Materials and methods

Cells and viruses

We grew the MT4 line of CD4 positive lymphocytes in suspension culture ($3–5 \times 10^6$ cells per ml) in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine and antibiotics as described) [5]. We worked with a number of clinical isolates, obtained

from patients prior to antiviral therapy by co-culture of patient peripheral blood lymphocytes with cord blood lymphocytes [2] and with the HIV-III_B laboratory strain of HIV-1, kindly provided by Dr. R. C. Gallo, National Institutes of Health, Bethesda, MD, U.S.A. Viruses were propagated on MT4 cells and stored at −70 °C [5]. Serum samples were obtained from HIV-infected asymptomatic donors and uninfected healthy controls.

Drugs

2′-3′-dideoxyinosine (ddI) and 2′,3′-dideoxy-3′-didehydrothymidine (d4T) were obtained from Bristol-Myers-Squibb (Wallingford, CT, U.S.A.). 3′-azido-3′-deoxythimidine (AZT; zidovudine) was obtained from Burroughs-Wellcome Inc., Research Triangle Park, NC. 2′,3′-dideoxycytidine (ddC) was purchased from Sigma Chemicals Inc., St. Louis, MO, U.S.A. The racemic mixture of the (−) and (+) enantiomers of 2′-deoxy-3′-thiacytidine, known as BCH-189 [22] was provided by BioChem Pharma Inc., Montreal, Canada, which also provided the (−) enantiomer, known as 3TC [20]. Interferon-2α (IFN-2α) was a gift of Schering Inc., Pointe-Claire, Que, Canada.

Selection of drug-resistant variants of HIV-1 by culture passage

MT-4 cells were pre-incubated for 30 minutes with sub-effective doses of compound (at or below the usual EC_{50} in each case) and were subsequently infected with HIV-1, using a multiplicity of infection of 0.01 tissue culture infectious doses-50% ($TCID_{50}$) per cell. Following a three hour absorbtion, the cells were washed and maintained in tissue culture medium at the same concentration of drug as used during both pre-incubation and infection. Medium changes were carried out twice weekly; each medium replacement contained gradually increasing drug concentrations as described [5]. Culture fluids (0.5 ml) from each round of viral replication were used to infect fresh MT4 cells [5]. Cultures were monitored on a regular basis for production of both viral reverse transcriptase activity and viral p24 antigen as described [2]. Polymerase chain reaction (PCR) methodology was used to detect previously described RT mutations that account for HIV drug resistance as previously described [7, 8, 12, 14, 20, 23].

Results

In vitro selection of drug-resistant HIV variants

MT-4 cells infected with either HIV-III_B or clinical isolate 187 were cultured in the presence of increasing concentrations of each of AZT, d4T, ddI, and ddC as previously described [5]. Initial drug concentrations were 0.018 μM for AZT, 0.025 μM for d4T, 19 μM for ddI and 0.75 μM for ddC. HIV variants present after 8 passages (4 weeks) demonstrated nearly 100-fold resistance to AZT, 30-fold resistance to d4T, and 10–20 fold resistance for each of ddI and ddC, in comparison to parental strains (Table 1). Step-wise increases in levels of drug resistance are shown for each of passage levels 4, 6 and 8, at which times such assessments were carried out.

The results of Table 2 present data on nucleoside susceptibilities of viruses selected for resistance in AZT, ddI, or ddC, and include replication studies performed with each of the above compounds as well as d4T and 3TC. The viruses that were resistant to ddI were resistant as well to ddC, although the reverse was not true. AZT-resistant viruses remained fully susceptible to each

Table 1. Susceptibility of HIV variants at different passage levels to anti-viral nucleosides in single drug selection protocols[a]

Passage no	Drug used in selection (μM)				EC_{50} (μM)[b]							
	AZT	d4T	ddI	ddC	HIV-III_B				Clinical isolate 187			
					AZT	d4T	ddI	ddC	AZT	d4T	ddI	ddC
1	0.018	0.25	19	0.75	0.01	0.45	10.2	0.41	0.013	0.41	10.5	0.45
2	0.025	0.5	34	1.1								
3	0.0325	1.8	85	2.5								
4	0.0375	3.7	169.5	5.2	0.25	3.5	96.5	2.3	0.32	4.0	104.2	2.8
5	0.75	7.5	254	11.5								
6	1.5	15	339	11.5	0.71	11	170.4	4.8	0.85	14	188.2	5.8
7	1.9	19	424	25								
8	1.9	19	424	25	0.92	15	215.4	5.8	1.2	17	227.5	7.2

[a] HIV variants were generated by in vitro selection using the HIV-III_B laboratory strain and clinical isolate 187 obtained from a patient prior to nucleoside therapy

[b] EC_{50} values were determined by measurement of p24 Ag levels in culture fluids

Table 2. Patterns of drug resistance using HIV variants selected for resistance to AZT, ddI or ddC

Variant	EC_{50} (μM)				
	AZT	ddI	ddC	d4T	3TC
$HIV\text{-}III_B$	0.01	10.2	0.41	0.45	0.47
AZT-resistant $HIV\text{-}III_B$	0.9	10.4	0.43	2.1	0.61
ddI-resistant $HIV\text{-}III_B$	0.018	215.2	5.1	0.39	2.1
ddC-resistant $HIV\text{-}III_B$	0.015	18.4	5.8	0.47	4.9
Clinical isolate 187	0.013	10.5	0.45	0.41	0.54
AZT-resistant 187	1.4	10.2	0.45	0.6	0.49
ddI-resistant 187	0.02	237.2	1.3	0.43	2.9
ddC-resistant 187	0.018	15.2	8.2	0.41	5.7

EC_{50} values were calculated on the basis of viral reverse transcriptase (RT) activity in culture fluids. Care was taken to ensure that peak levels of RT activity were being monitored in each case

of ddI and ddC, although some cross-resistance against d4T was demonstrated, confirming previous findings [19]. Variants selected for resistance to either ddI or ddC remained susceptible to AZT, but were cross-resistant to 3TC.

In vitro selection of multiple HIV drug resistance

We next investigated whether multiple drug resistance could also be generated by in vitro selection. We began with AZT-resistant viruses, which were propagated on MT-4 cells in the presence of increasing concentrations of ddI or ddC, respectively, as described above and in Table 1. Fresh MT-4 cells were included at each passage, as in the case of selection for resistance against a single drug, and viruses were assessed for susceptibility to each of AZT, ddI, and ddC. Through 10 such passages, we were able to generate variants that retained 90-fold resistance to AZT and also possessed 10–15 fold resistance to either ddI or ddC (Table 3). It is interesting that no diminution in levels of resistance against AZT was detected during this selection procedure. Furthermore, we observed that no further resistance against ddI could be generated after 8 weeks of selection, while resistance against ddC increased marginally between 8 and 10 weeks.

To investigate whether triple drug resistance could be independently generated, we began with AZT-resistant viruses, which were propagated on MT-4 cells in the presence of increasing concentrations of ddI or BCH-189, respectively. This was followed by a subsequent selection in the presence of d4T. The data of Table 4 indicate that multiple drug resistance could indeed be demonstrated using this approach.

We also examined these multiply resistant HIV variants by polymerase chain reaction (PCR) to determine whether they contained mutation sites

Table 3. Drug susceptibilities of AZT-resistant variants at different passage levels in the presence of ddI and ddC[a]

Variant	Drug pressure during passage	EC_{50} (μM) at passage														
		0			4			6			8			10		
		AZT	ddI	ddC	AZT	ddI	ddC	AZT	ddI	ddC	AZT	ddI	ddC	AZT	ddI	ddC
AZT-resistant $HIV\text{-}III_B$	ddI	0.9	10.4	0.41	0.88	52.4	0.63	0.82	88.5	0.84	0.79	131.8	1.7	0.85	120.1	1.9
AZT-resistant $HIV\text{-}III_B$	ddC				0.86	13.4	1.3	0.84	12.2	2.3	0.88	13.1	5.4	0.91	11.5	6.1
AZT-resistant strain 187	ddI	1.4	10.2	0.42	9.8	61.5	0.81	1.2	92.4	1.4	1.1	109.7	2.6	1.2	105.6	2.9
AZT-resistant strain 187	ddC				1.1	10.1	1.5	9.7	12.5	4.1	1.4	11.7	6.6	1.1	10.8	7.3

[a] AZT-resistant variants were selected over 10 passages for subsequent resistance to ddI or ddC as described in Materials and methods. EC_{50} values were calculated from levels of p24 Ag in culture supernatants

Table 4. Drug susceptibilities of AZT-resistant and other resistant variants after passage in the presence of other nucleosides[a]

Variant	Initial mutation at site	Drug pressure during second passage series	Subsequent mutation	Drug pressure during third passage series	EC_{50} (μM) after third passage series				
					AZT	ddI	ddC	d4T	BCH-189
AZT-resistant $HIV\text{-}III_B$	41, 215	ddI	74	–	0.9	120	0.5	0.5	0.8
AZT-resistant $HIV\text{-}III_B$	41, 215	ddI	74	d4T	1.2	134	1.6	8.5	2.9
AZT-resistant $HIV\text{-}III_B$	41, 215	BCH-189	184	–	0.85	15.6	3.8	0.6	187
AZT-resistant $HIV\text{-}III_B$	41, 215	BCH-189	184	d4T	1.1	12.8	7.1	12.5	50.2
ddI-resistant $HIV\text{-}III_B$	74	d4T	na[b]	–	0.2	114	1.4	6.9	2.7
BCH-189-resistant $HIV\text{-}III_B$	184	–			0.03	47	2.7	0.8	480.00

[a] AZT-resistant or ddI-resistant variants were selected over 10 passages for subsequent resistance to other compounds as described in Materials and methods. EC_{50} values were calculated from RT activity in culture supernatants

[b] *na* Not analyzed

Table 5. Susceptibility of multiply drug-resistant HIV variants to nucleosides after propagation in the absence of drug pressure[a]

Variant	EC_{50} (μM) after further growth in the absence of drug for					
	0 months			2 months		
	AZT	ddI	ddC	AZT	ddI	ddC
Multiply-resistant HIV-III_B	0.81	123.4	1.4	0.84	115.7	1.4
Multiply-resistant HIV-III_B	0.94	15.2	6.3	0.91	12.8	5.5
Multiply-resistant strain 187	0.98	120.5	1.9	1.1	119.2	2.3
Multiply-resistant strain 187	1.2	11.4	6.8	1.2	14.5	6.2

[a] Multiply drug-resistant variants of HIV-1 were propagated on MT-4 cells in the absence of drug. Susceptibility assays were performed after 1 and 2 months, respectively, in the absence of drug pressure

previously identified as responsible for diminished sensitivity to AZT [12, 14], ddI [23], and BCH-189 [7, 20]. The results of Table 4 show that viruses resistant to AZT contained codon alterations at sites 41 and 215; ddI-resistant viruses possessed mutation 74 and BCH-189 resistant viruses contained mutation 184. Additional passage in nucleoside analogs could be shown to result in acquisition of new mutations in the case of both ddI and BCH-189.

Stability of the multiple drug resistance phenotype

Multiply resistant HIV variants that had been generated in tissue culture were further propagated on MT-4 cells in the absence of drug for 2 months. After this time, viruses were assessed for ability to replicate in the presence of anti-viral nucleosides, as assessed by p24 Ag levels in culture fluids. No significant loss of drug resistance was detected (Table 5).

Use of combinations of nucleosides and neutralizing antisera or interferon-2α

We further determined whether drug resistance would emerge when a single compound was used in our selection protocol together with high dilutions of virus-neutralizing antisera. As controls, we employed combinations of various nucleosides simultaneously or combinations of single nucleosides with interferon-2α. The results of Fig. 1 demonstrate that the addition of sub-titer concentrations of virus-neutralizing sera to the AZT and ddI concentration gradients described in Table 1 was non-permissive for emergence of HIV variants resistant to either of these drugs. This effect could be further diluted through the use of even lower serum concentrations to permit the emergence of drug-resistant viruses.

The development of HIV drug resistance was also reduced when combinations of IFN-2α were used together with each of AZT, ddI and ddC in our selection

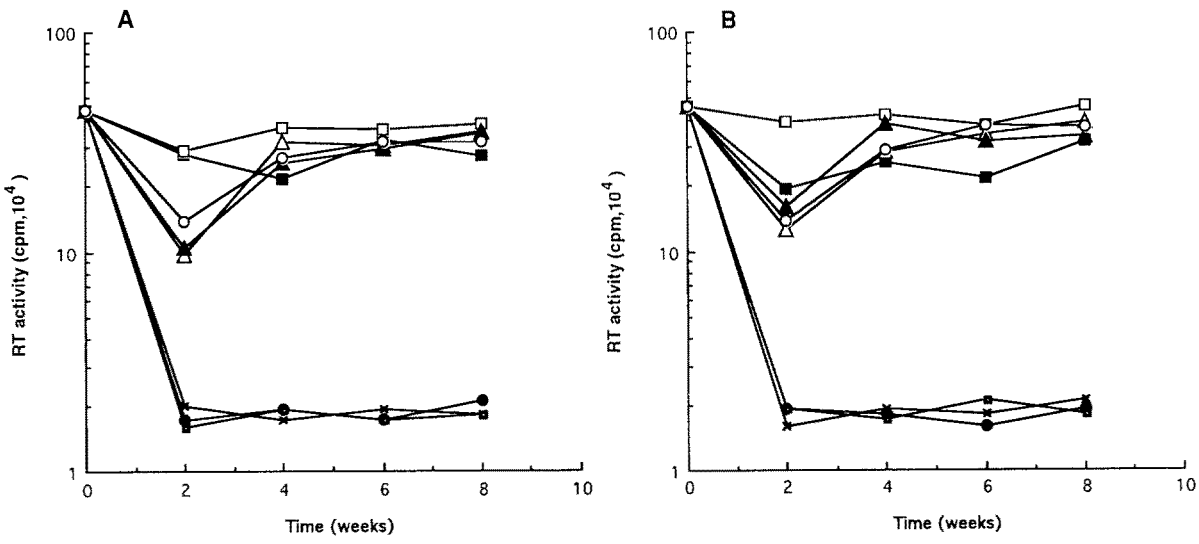

Fig. 1. Virus production assessed by RT activity in MT-4 cells infected with HIV-III$_B$ (**A**) and clinical isolate 187 (**B**) in the presence of 1:1 000 diluted patient serum (□), 1:200 diluted patient serum (■), 0.01 μM AZT plus 1:1 000 diluted patient serum (△), 19 μM ddI plus 1:1 000 diluted patient serum (▲), 0.75 μM ddC plus 1:1 000 diluted patient serum (○), 0.01 μM AZT plus 1:200 diluted patient serum (●), 19 μM ddI plus 1:200 diluted patient serum (**x**), 0.75 μM ddC plus 1:200 diluted patient serum (■)

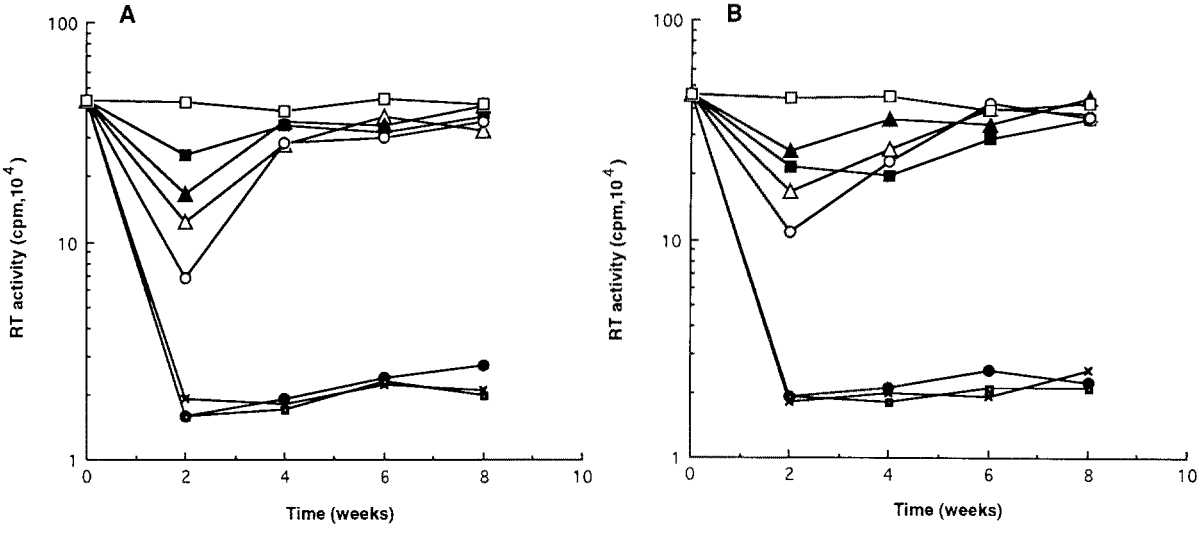

Fig. 2. Virus production assessed by RT activity in MT-4 cells infected with HIV-III$_B$ (**A**) and clinical isolate 187 (**B**) in the presence of 10 IU IFN-α2 (□), 50 IU IFN-α2 (■), 0.01 μM AZT plus 10 IU IFN-α2 (△), 19 μM ddI plus 10 IU IFN-α2 (▲), 0.75 μM ddC plus 10 IU IFN-α2 (○), 0.01 μM AZT plus 50 IU IFN-α2 (●), 19 μM ddI plus 50 IU IFN-α2 (**x**), and 0.75 μM ddC plus 50 IU IFN-α2 (■)

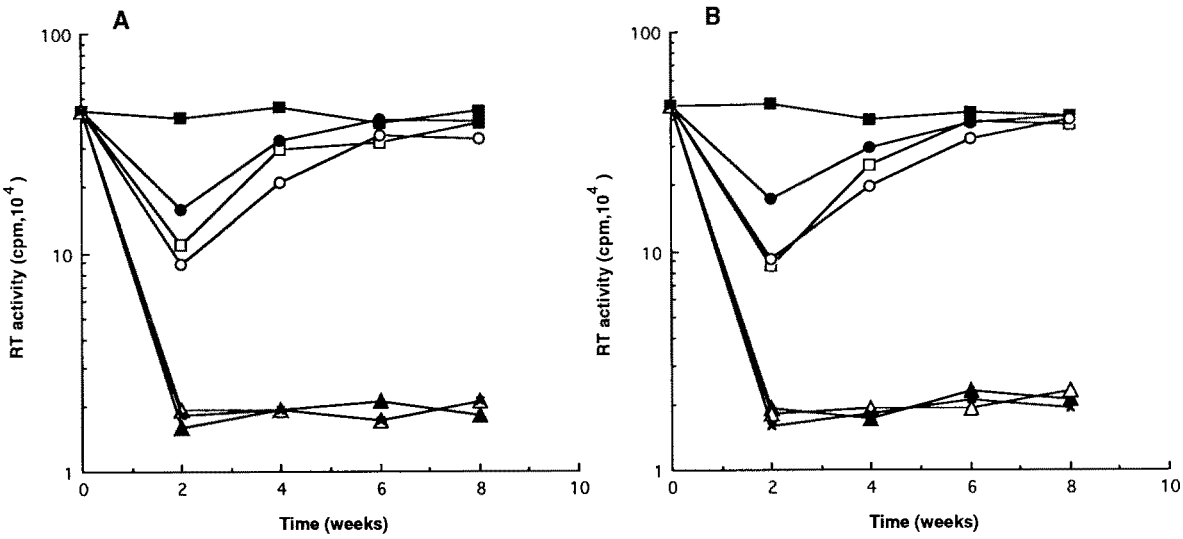

Fig. 3. Viral replication assessed by RT activity in MT-4 cells infected with HIV-III$_B$ (**A**) and clinical isolate 187 (**B**). Infected MT-4 cells were cultured in the absence of compound (■) or in the presence of 0.01 μM AZT (□), 19 μM ddI (●), 0.75 μM ddC (○), 0.01 μM AZT plus 19 μM ddI (▲), 0.01 μM AZT plus 0.75 μM ddC (△), 19 μM ddI plus 0.75 μM ddC (**x**)

protocols (Fig. 2). The synergistic effects obtained with regard to non-emergence of resistance to each of AZT, ddI and ddC could no longer be documented when very low concentrations of IFN-2α were utilized, indicating that this effect was concentration-dependent. However, the concentrations of IFN-2α that effected synergy with either AZT or ddI in terms of non-emergence of drug resistance were below those able to achieve a significant anti-viral effect in the absence of nucleoside antagonists of viral RT activity [6].

Combinations of various sub-threshold concentrations of nucleosides, including AZT plus ddI, AZT plus ddC, and ddI plus ddC, were likewise non-permissive for emergence of drug-resistant variants (Fig. 3). Of course, these data indicate that a first round of viral replication could not be achieved when certain drug combinations were present. Since some level of viral multiplication would be necessary for selection of drug resistance, these data do not exclude the possibility that other conditions might permit the emergence of resistant variants.

Discussion

The major findings of this paper relate to our ability to have generated strains of HIV-1 that are multiply resistant to AZT, ddI and ddC through in vitro culture and drug pressure procedures. Previous work showed that such resistance against individual nucleosides can develop in tissue culture over periods between 2–8 weeks. Combinations of nucleosides, even at sub-effective concentrations, or use of IFN-2α in concert with nucleosides, did not permit

the emergence of resistant variants, presumably due to synergy [6]. Of course, generalization with regard to patterns of cross-resistance among different isolates cannot be made on the basis of the limited numbers of samples reported here. Others have also shown that the use of triple drug combinations can yield multiple resistance, using protocols similar to those described here [13].

The drug-resistant variants derived in the current study possessed 50–100-fold resistance against AZT and 10–25-fold resistance against either ddI or ddC. These viruses displayed a stable multiple drug resistance phenotype, when grown in the absence of drug for 1–2 months. Non-reversion to a drug-sensitive phenotype probably reflects the stability of the mutations at specific sites in the HIV-1 pol gene that are responsible for drug resistance. Extensive viral passage in the absence of drug did not yield progeny of diminished drug sensitivity. Mutations at codons 41 and 215, among other sites, confer resistance to AZT, while mutations at codons 74 and 184 are associated with resistance to both ddI and ddC [8, 23]. Our PCR analyses showed that the multiply resistant viruses studied (AZT, ddI, ddC) contained mutations at codons 74 and 184 as well as at codons 41 and 215. The mutations at positions 74 and 184 probably resulted from passage of AZT-resistant species in ddI and ddC.

The AZT-resistant variants selected possessed similar genotypes to those isolated from patients under prolonged therapy with this drug [5, 15]. Other workers have noted that the same order of pol gene mutations seen in patients is likely to occur in vitro [1, 14]. We used relatively fresh isolates of HIV-1, selected after fewer than 10 culture passages, to minimize the extent of heterogeneity in reverse transcriptase.

We also demonstrated that no emergence of drug resistance occurred when single drugs were used in concert with either IFN-2α or high dilutions of virus-neutralizing antisera. Significantly, the concentrations of IFN-2α employed were as low as 10 IU/ml, far below those previously shown to possess an anti-viral effect. Previous studies demonstrated synergy between AZT and IFN-2α with regard to inhibition of acute HIV replication in tissue culture, but viral breakthrough was ultimately observed even when IFN concentrations of 50 IU were employed in the presence of AZT. The viruses which emerged from such investigations were wild-type and could be inhibited by both AZT and IFN-2α during subsequent replication cycles. In vitro selection for resistance may be a more sensitive method than acute infection for demonstrating cooperativity between interferon and nucleosides. Similar cooperative effects were demonstrated between nucleosides and neutralizing antibodies, which were shown to be effective in dose-dependent fashion.

HIV-1 variants that display multiple drug resistance have been isolated from patients sequentially treated with different drugs [23]. Our study demonstrates that such viruses can be selected more easily in vitro under conditions of sequential rather than simultaneous drug exposure. This finding is consistent with reports of the persistence of AZT resistance-conferring mutations in plasma, even after treatment with ddI and appearance of resistance

to the latter drug [21]. The concept of combination simultaneous therapy should be further investigated.

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