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Didanosine (ddI) and zidovudine (ZDV) susceptibilities of human immunodeficiency virus (HIV) isolates from long-term recipients of ddI

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Summary

Thirty HIV isolates, obtained from 15 patients before and after receiving single drug therapy with didanosine (ddI), were examined for sensitivity to ddI and zidovudine (ZDV) using a peripheral blood mononuclear leukocyte (PBML)-based assay. Fourteen of the patients had ARC, one had AIDS and 12 had received previous therapy with ZDV. After a median of 1 year of ddI therapy, isolates were significantly less sensitive to ddI than were isolates obtained prior to therapy ($P = 0.03$). A decrease in ddI sensitivity was observed in ten of the 15 isolate pairs. In contrast to ddI susceptibilities, sensitivity to ZDV increased over the same period of time ($P = 0.03$). Additional isolates were obtained from four patients who received ddI monotherapy for 2 years. Three of these isolates demonstrated no change in ddI sensitivity compared to baseline. No correlation could be made in this study between development of decreased ddI sensitivity and serum p24 levels, CD4 counts, or clinical outcome. Decreased ddI sensitivity occurs frequently among HIV isolates obtained from long-term recipients of ddI. This decreased sensitivity is modest in degree and is of unknown clinical significance.

Didanosine (ddI); Zidovudine (ZDV); HIV resistance

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Introduction

Development of resistance to nucleoside analogs among clinical isolates of human immunodeficiency virus (HIV) was first described for zidovudine (ZDV) in 1989 (Larder et al., 1989a). Resistance to ZDV has been observed most frequently among isolates obtained from patients with advanced HIV disease who have received the drug for prolonged periods of time (Larder et al., 1990; Rooke et al., 1989; Richman et al., 1990; Boucher et al., 1990). ZDV resistance has been associated with the development of specific mutations in the virus reverse transcriptase (RT) gene (Larder and Kemp, 1989b; Kellam et al., 1992). These mutations appear sequentially, and higher levels of resistance are associated with multiple mutations (Boucher et al., 1992; Larder et al., 1991). Resistance of HIV isolates to nucleoside analogs, other than ZDV, has been described less frequently. HIV-1 isolates resistant to 2′,3′-dideoxyinosine (didanosine, ddI) have been induced by selection in vitro, and have also been detected among clinical isolates obtained from small numbers of patients (Gao et al., 1992; St. Clair et al., 1991; Japour et al., 1991). Interestingly, the development of decreased susceptibility to ddI has been associated with increased ZDV sensitivity. A single, specific RT mutation at position 74 has been associated with decreased susceptibility of HIV to ddI (St. Clair et al., 1991). Clinical isolates with this mutation have been described only in the setting of pre-existing mutations conferring ZDV resistance.

In the studies described in this report, we utilized a peripheral blood mononuclear leukocyte (PBML)-based assay to measure susceptibility of HIV isolates to antivirals, and observed development of decreased sensitivity to ddI among isolates obtained from 15 patients after treatment with ddI for a median of 1 year. We also noted that resistance to ZDV decreased during this period of ddI monotherapy. Typical ZDV-associated RT mutations were detected in isolates obtained from patients who had previously received ZDV, and the mutation at position 74 was seen in isolates with decreased sensitivity to ddI.

Materials and Methods

Patients and study design

HIV isolates which were evaluated in this study were all obtained from participants in a National Institute of Allergy and Infectious Diseases (NIAID) AIDS Clinical Trials Group (ACTG) phase I study of ddI (ACTG 064). This trial was conducted at the University of Rochester and at New York University, and has been described in detail elsewhere (Lambert et al., 1990; Dolin et al., 1990). Isolates evaluated in the present study were all obtained from patients enrolled at Rochester. At the time of study enrollment, HIV isolates were recovered from all study participants using primary PBML cultures. Patients then received ddI according to a dose escalation protocol for periods of up to 2 years.

Virus isolation and preparation of virus stocks

HIV isolation from patient specimens was performed according to a modification of a protocol developed by the ACTG Viral Research Laboratory (VRL) (Hollinger et al., 1992). Fresh PBML from HIV seronegative subjects were separated from heparinized blood using Ficoll-Hypaque gradients. These 'donor' cells were incubated for 3 days in the presence of IL-2 and PHA, and were then co-cultivated with patients' PBML for up to 28 days. Antiviral drugs were not added to the culture media. Aliquots of supernatants were removed at 3–4 day intervals, stored at −70°C, and subsequently assayed for p24 content. p24 Measurements were made using the Abbott kit (Abbott Laboratories, Diagnostics Division, Abbott Park, IL), with the modification that the standard curve was prepared using the HIV antigen reference standard provided by the VRL.

Virus stock pools were established for each HIV isolate in primary cultures of PBML using methods analogous to those employed for initial virus isolation. No antiviral drugs were added to the cultures. After approx. 10 days of culture, aliquots of supernatants were stored at −70°C and later titrated using the Reed-Muench method (Reed and Muench, 1938), with p24 production as an endpoint.

Virus susceptibility testing

Susceptibility testing of both pre- and post-therapy isolates obtained from each patient was always conducted in the same assay, using PBML from the same donor.

PBML cultures consisting of 10^6 cells were established in 1 cc vol in 24-well polystyrene plates (Costar Corp., Cambridge, MA). Amounts of ddI (provided by Bristol-Myers Squibb, Wallingford, CT) or ZDV (supplied by Burroughs-Wellcome Company, Research Triangle Park NC) selected to achieve desired concentrations were added 3 days later. At the same time, cultures were inoculated with 50-100 $TCID_{50}$, as determined by the Reed-Muench method, of the HIV isolate to be tested. All cultures were performed in duplicate. On days 4 and 8, one-half of the culture medium was replaced with fresh medium containing amounts of antiviral drugs necessary to maintain initial concentrations. On day 8, fresh donor cells were also added. On day 12, aliquots of supernatants were removed, stored, and later assayed for p24 content. When necessary to obtain an exact measurement, serial dilutions of separate aliquots of supernatants were also assayed for p24 content.

Determination of minimum inhibitory concentration (MIC)

ddI Concentrations of 5 μM, 10 μM, 50 μM and 100 μM, and ZDV concentrations of 0.01 μM, 0.1 μM, 1.0 μM and 10.0 μM, were employed. The MIC was defined as the drug concentration at which the p24 content in the test culture was reduced by 90% or greater as compared to untreated virus cultures.

Statistics

MIC values were calculated from an exponential regression model of p24 antigen concentration as a function of drug concentration (CSS: STATISTICAL software; Statsoft, Tulsa, OK). The fit of the model was assessed by the proportion of variance explained, with 90% or greater considered acceptable.

The Wilcoxon signed rank procedure was used to compare to zero the differences between post- and pre-treatment MICs (Minitab, version 7.2 software; Minitab, State College, PA). All P values are two-tailed, and $P < 0.05$ is considered to be significant. Approximate 95% confidence intervals were also calculated.

DNA sequencing

DNA sequencing was accomplished by first amplifying selected regions of the pol gene using the polymerase chain reaction (PCR). Base sequences of PCR products were then determined by the dideoxy method using Taq polymerase (fmol™ sequencing system; Promega, Madison, WI) (Sanger et al., 1977; Sambrook et al., 1989; Murray, 1989; Saluz and Jost 1989; Carothers et al., 1989).

HIV isolates of interest were passaged in PBML cultures and proviral DNA was extracted using SDS and proteinase K according to published procedures (Sambrook et al., 1989). Using previously described locations of relevant mutations in the RT gene (Larder and Kemp, 1989; St. Clair et al., 1991), two regions were chosen as target sequences. One region (Region I) consisted of 342 bp and encompassed codons 41, 67, 70, 74, and the other (Region II) was 147 bp long and included codons 215, 219. A set of two pairs of oligonucleotide primers was designed to amplify each of the target sequences and a nested set PCR was performed for each.

Primer sequences were:

Region I:	Outer Pair:	5′-CAGTAAAATTAAAGCCAGG-3′
		5′-CATTGTACTGATATCTAATC-3′
	Inner Pair:	5′-GATGGCCCAAAAGTTAA-3′
		5′-GCATCACCCACATCC-3′
Region II:	Outer Pair:	5′-CAATACATGGATGATTTG-3′
		5′-ACTGTCCATTTATCAGG-3′
	Inner Pair:	5′-GATGATTTGTATGTAGG-3′
		5′-TCAGGATGGAGTTCATA-3′

The first round of the nested set PCR was performed on 5 μl of extracted DNA using unlabelled outer primers for each of the two target sequences.

For the second round PCR, which also includes the sequencing reaction, 2 μl of the first round PCR products were used to further amplify the target

TABLE 1

Characteristics of patients from whom HIV isolates were obtained

Number with ARC/AIDS	14/1
Men/women	13/2
Median age in years (range)	38 (25–62)
Number with previous ZDV experience	12
Median duration of ZDV therapy in weeks (range)	36 (10–90)
Median duration of ddI therapy in weeks (range)	56 (40–72)

sequences of interest using γ-^{32}P 5′ end-labelled inner primers along with sequencing grade Taq DNA polymerase (fmol^{TM}, Promega, Madison, WI). The sequencing mixtures were then loaded on 8% polyacrylamide gels and electrophoresed. DNA bands were visualized by autoradiography.

Results

Patient characteristics

Of the 15 patients who participated in this study, 1 had AIDS and the other 14 had ARC as determined by Centers for Disease Control (CDC) criteria (Table 1). Most of the study participants were men, and all but three had previously been treated with ZDV for a median of 36 weeks. The first isolate of each isolate pair examined in this study was obtained prior to the initial dose of ddI. The second isolate was obtained after administration of ddI for approx. 1 year. Additional isolates were obtained after administration of ddI monotherapy for periods of up to 2 years.

Sensitivity of HIV isolates to ddI

HIV isolates obtained after a median of 56 weeks of ddI monotherapy were significantly less sensitive to ddI than were pre-therapy isolates (Table 2). The median ddI MIC measured before therapy was 6.5 μM, and the median ddI MIC after therapy was 13.3 μM. The median difference between post- and pre-therapy MIC values was 9.4 μM ($P = 0.03$; Confidence Interval = 1.0, 19.6). In 10 of the 15 isolate pairs, ddI MICs rose over the study period (Fig. 1). The magnitude of the MIC rises ranged from <2- to 14-fold with a median of 2.5-fold.

Additional isolates were available from four of the patients (numbers 1, 2, 7 and 9 – see Fig. 1 and Table 2) who received ddI monotherapy for a median of 96 weeks. Of these four, only patient 1 had not received previous ZDV therapy. These four isolates had a median ddI MIC of 7.2 μM with a range of 5 μM to 21 μM.

Sensitivities of HIV isolates to ZDV

In contrast to ddI, post-therapy HIV isolates were more sensitive to ZDV than were pre-therapy isolates (Table 3). The median difference between pre-

TABLE 2

ddI susceptibilities of HIV isolates obtained before and after one year of ddI monotherapy

Patient*	MIC μM	
	Pre-therapy isolate	Post-therapy isolate
1	13.6	<5.0
2	<5.0	<5.0
3	10.7	<5.0
4	7.2	>100.0
5	44.1	62.7
6	6.5	31.2
7	<5.0	13.3
8	12.7	<5.0
9	<5.0	<5.0
10	5.8	14.0
11	13.6	33.3
12	25.2	51.4
13	<5.0	24.4
14	<5.0	12.7
15	5.0	7.0
Median	6.5	13.3**

*Patient numbers correspond to those in Fig. 1.
**Post-therapy MIC values are significantly greater than pre-therapy MIC values.
$P = 0.03$, by Wilcoxon signed-rank test.

and post-therapy MIC values was 0.10 μM ($P = 0.01$; Confidence Interval = 0.25, 0.01). Eight of the pre-treatment isolates had ZDV MICs of 0.1 μM or greater.

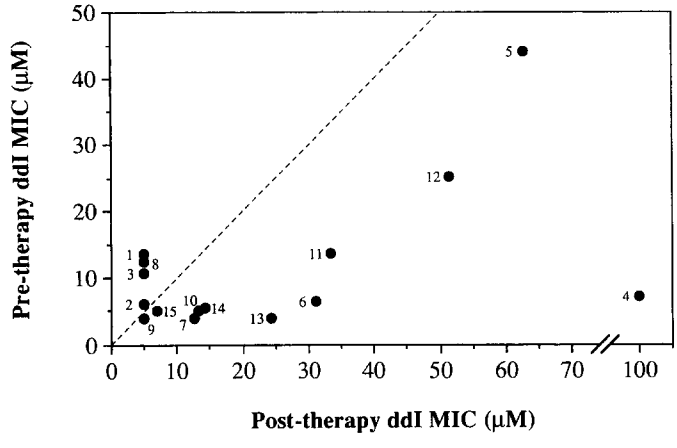

Fig. 1. Results of ddI sensitivity testing. Pre-therapy ddI MICs, indicated on the vertical axis, are plotted against post-therapy values, indicated on the horizontal axis. Circles to the right of the line represent isolate pairs in which the post-therapy MIC is higher than the pre-therapy MIC. Patient numbers, which correspond to those listed in Table 2, are indicated by numerals adjacent to circles. Patients 1, 5 and 12 had not received prior ZDV therapy.

TABLE 3

ZDV susceptibilities of HIV isolates obtained before and after 1 year of ddI monotherapy

	MIC (μM)	
	Median	Range
Pre-therapy isolates	0.12*	0.01–10.0
Post-therapy isolates	0.01	0.01–0.27

*Pre-therapy MIC values are significantly greater than post-therapy MIC values. $P = 0.03$, by Wilcoxon signed-rank test.

DNA sequencing

Results of DNA sequencing studies performed on six selected isolates obtained from three patients are summarized in Table 4. Patient No. 1 was an AIDS patient who had received ZDV therapy for 11 months prior to enrollment into study. An isolate obtained prior to ddI therapy demonstrated moderate resistance to ZDV and a mutation at position 215 (Thr→Tyr) was detected. After 1 year of ddI therapy, both the ZDV and ddI MICs had increased, and mutations were detected at positions 41 (Met→Leu) and 74 (Leu→Val), as well as 215. Patient No. 2 had ARC, and had received ZDV for 8 months prior to study enrollment. An HIV isolate obtained prior to enrollment was sensitive to both ZDV and ddI, and reverse transcriptase mutations previously associated with ZDV and ddI resistance were not detected. After 1 year of ddI therapy, an HIV isolate obtained from this patient remained sensitive to ZDV, but the ddI MIC had risen by more than 5-fold. A mutation at position 74 (Leu→Val) was detected in this isolate. Isolates obtained from patient No. 3 prior to study enrollment and after 68 weeks of ddI monotherapy were susceptible to both ZDV and ddI, and mutations associated with nucleoside resistance were not detected.

Serum p24 antigen levels and CD4 counts

Eight of the 15 patients had detectable p24 antigen present in their sera prior to ddI therapy. In all instances, levels of p24 decreased within 2 weeks after the

TABLE 4

Antiviral susceptibilities and reverse transcriptase mutations of selected HIV isolates obtained before and after ddI therapy

Patient	Prior ZDV use	Week of ddI therapy	ZDV MIC (μM)	ddI MIC (μM)	Reverse Transcriptase mutations*					
					41	67	70	74	215	219
1	11 months	0	0.19	13	–	–	–	–	m	–
		48	0.89	39	m	–	–	m	m	–
2	8 months	0	0.01	<5	–	–	–	–	–	–
		56	0.01	24	–	–	–	m	–	–
3	None	0	0.01	14	–	–	–	–	–	–
		68	0.02	<5	–	–	–	–	–	–

* m, mutant; –, wild type.

start of therapy. In six patients, serum p24 levels rose after 6 months of study. In eleven patients, CD4 counts increased or remained stable during ddI therapy. In the other four individuals, CD4 counts decreased during the study period. Neither increasing levels of serum p24 antigen, nor falling numbers of CD4 cells were correlated with the development of decreased ddI sensitivity during this study.

Discussion

The studies described in this report demonstrate the development of decreased sensitivity to ddI of HIV isolates obtained from 15 HIV-infected patients treated with ddI for a median of 1 year. This decreased sensitivity was modest in degree, but was detected among post-therapy isolates obtained from about two thirds of patients. DNA sequencing studies demonstrated the appearance of a mutation at position 74 among isolates which developed decreased sensitivity to ddI. These observations are consistent with the report of St. Clair et al., who demonstrated, using site-directed mutagenesis techniques, that this mutation confers decreased sensitivity to ddI (St. Clair et al., 1991). In addition, our study demonstrates that HIV isolates obtained from ddI recipients may develop this mutation in the absence of pre-existing ZDV-associated mutations. The clinical significance of this decrease in ddI sensitivity is unknown. In this small group of patients, no obvious relationship between decreased sensitivity and p24 levels, CD4 counts, or clinical course was observed.

In this study, HIV isolates which were examined were obtained primarily from patients with ARC. In longitudinal studies of HIV isolates examined for susceptibility to ZDV, the magnitude of resistance and the frequency with which resistance develops are both correlated directly with severity of illness as well as with duration of therapy (Richman et al., 1990). Thus, patients with AIDS who are treated with ZDV for prolonged periods are much more likely to develop ZDV-resistant strains of HIV, than are asymptomatic HIV-infected patients treated for shorter periods. In addition, RT mutations associated with ZDV resistance appear sequentially, and multiple mutations appear to produce higher levels of resistance than do single mutations (Kellam et al., 1992; Boucher et al., 1992). Whether similar observations will be made with future studies of ddI is unknown. With the recent licensure of ddI, and the widespread use of the drug in populations with severe immunodeficiency, HIV isolates from patients with AIDS who have received ddI for prolonged periods are becoming available. These isolates will need to be examined carefully to determine whether or not the magnitude and frequency of resistance to ddI will increase significantly over that which we have observed in the present study. Such investigations should include both phenotypic and genotypic studies to detect and characterize additional RT mutations which might be associated with ddI resistance.

In addition to demonstrating the development of decreased sensitivity to ddI, the present study also examined the sensitivity of sequentially obtained isolates during ddI monotherapy to ZDV. In this group of 15 patients, 12 had received previous ZDV therapy, and the median ZDV MIC at study entry was 0.12 μM, a level generally agreed upon to represent moderate resistance to ZDV. This observation is similar to that reported previously by others who have examined ZDV susceptibilities of HIV isolates from analogous groups of patients (Larder et al., 1989a; Rooke et al., 1989; Boucher et al., 1992). In the present study, we also observed that after 1 year of monotherapy with ddI, isolates from these patients were more sensitive to ZDV than were isolates obtained from these patients prior to ddI treatment. A similar observation was made by St. Clair et al., (St. Clair et al., 1991), who demonstrated that the mutation at position 74 which is associated with decreased ddI susceptibility appears to confer increased sensitivity to ZDV in the setting of existing RT mutations associated with ZDV resistance. We encountered a similar situation with patient 1 (Table 4), although in that instance the recently described mutation at position 41 (Kellam et al., 1992) appeared, and an increase in the ZDV MIC was observed. Whether the position 74 mutation can completely explain the observations of increased ZDV susceptibility made in the present study is unknown. An alternative explanation might be that the absence of ZDV therapy for prolonged periods might lead to re-establishment of a predominantly ZDV sensitive population of viruses in which the RT mutations associated with resistance to ZDV are no longer present. Additional studies designed to characterize the RT gene from multiple isolates will be required to determine which of these mechanisms is responsible for our observations, or whether additional molecular mechanisms are involved.

The importance of nucleoside resistance in the clinical course of nucleoside-treated HIV-infected patients is incompletely understood, and is an area of extremely active interest and investigation. At least one recent study (Tudor-Williams et al., 1992), directly supports the hypothesis that development of ZDV resistance is associated with clinical deterioration. Recently completed studies conducted by the NIH ACTG comparing ZDV and ddI should further clarify the importance of in vitro ZDV resistance in the clinical response to treatment with this class of drugs. Another major area of investigation is directed towards evaluation of the potential impact of combination nucleoside therapy on development of nucleoside resistance. This question is currently being addressed by several studies. For example, an ongoing ACTG protocol is investigating the development of ZDV, ddI, and ddC resistance among a cohort of patients receiving monotherapy or combination therapy with these agents.

In summary, treatment of ARC patients with ddI for a 1 year period leads to the development of decreased ddI sensitivity among isolates obtained from the majority of treated patients. This decreased sensitivity is modest in degree, and is associated with the development of a mutation at position 74 in at least some isolates. Among patients previously treated with ZDV, HIV isolates with

moderate levels of ZDV resistance appear to become more susceptible to ZDV after 1 year of ddI monotherapy. Determination of the clinical significance of these observations will require carefully conducted, longitudinal studies of multiple HIV isolates obtained from large numbers of patients.

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