ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1992, p. 920–925
0066-4804/92/050920-06$02.00/0

Vol. 36, No. 5

Didanosine and Zidovudine Resistance Patterns in Clinical Isolates of Human Immunodeficiency Virus Type 1 as Determined by a Replication Endpoint Concentration Assay

GAVIN X. McLEOD,* JACQUELINE M. McGRATH, ELIZABETH A. LADD, AND SCOTT M. HAMMER

Division of Infectious Diseases, Department of Medicine, New England Deaconess Hospital and Harvard Medical School, Boston, Massachusetts 02215

Received 20 November 1991/Accepted 14 February 1992

Reports of in vitro resistance of human immunodeficiency virus type 1 (HIV-1) to zidovudine (AZT) have raised concerns about the development of resistance to other dideoxynucleosides in clinical use. To address this, we have developed a screening assay which supports the growth of clinical isolates and have applied this to a series of paired isolates from patients entered into a phase I trial of didanosine (DDI). Thirteen patients (10 with AIDS, 3 with AIDS-related complex) who had been exposed to AZT for a mean of 6.5 months (range, 1 to 13 months) were treated with DDI at 750 mg/day. Paired isolates were obtained pretherapy and after a mean of 58 weeks (range, 21 to 90) of DDI therapy by coculture of peripheral blood mononuclear leukocytes (PBLs) with phytohemagglutinin-stimulated donor PBLs. Isolates were passaged only one additional time in PBLs and then tested in parallel in a microtiter assay with phytohemagglutinin-stimulated donor PBLs as targets. PBLs were infected with 10^5 50% tissue culture infectious doses per 10^7 cells and exposed to DDI (1 to 50 μM) or AZT (0.01 to 100 μM), and supernatants were assayed for the HIV p24 antigen at 7 days postinfection. Control AZT-susceptible and resistant isolates were included. The median pre- and posttherapy DDI susceptibilities of the 13 pairs of isolates were 10.0 μM (range, 1 to 25 μM) and 17.5 μM (range, 2.5 to 50 μM), respectively ($P = 0.036$; Wilcoxon signed-rank test). Three patients with the most susceptible initial isolates (mean, 2 μM) had mean increases in CD4 cell numbers of 88/mm^3 during the first 12 weeks of treatment, compared with a mean increase of 19/mm^3 in 10 patients with isolates with DDI susceptibilities of >2.5 μM ($P = 0.02$; two sampled t test). No correlation between in vitro susceptibilities and changes in HIV p24 antigen levels in serum was noted. Median susceptibilities to AZT improved from 1.0 μM (range, 0.01 to 100 μM) to 0.3 μM (0.01 to 100 μM) in the paired isolates ($P = 0.008$; Wilcoxon signed-rank test). These studies thus indicate that (i) the susceptibility to DDI tends to mildly decrease with drug exposure; (ii) the susceptibility to AZT improves with time off AZT; (iii) baseline susceptibilities to DDI have a wide range, and the CD4 response may correlate with the initial susceptibility; and (iv) a PBL-based microtiter assay is useful for screening clinical isolates for dideoxynucleoside susceptibility profiles.

Since the initial report of in vitro zidovudine (AZT) resistance by Larder et al. in 1989 (12), there has been intense interest in defining the clinical significance of this finding (2, 10, 25, 27), its occurrence in treated patients at different stages (19), and whether the phenomenon will be seen with the other dideoxynucleosides in widespread clinical use, didanosine (DDI) and 2′,3′-dideoxycytidine. The importance of these questions has also focused attention on the development of in vitro assays to detect antiretroviral resistance in an efficient and cost-effective manner. Previously reported assay systems have been useful but have each demonstrated important limitations. For example, the HeLa-CD4-based assays support the growth of only approximately 30% of clinical isolates (2, 4, 5, 12), the direct isolation of patient strains in dideoxynucleoside-containing medium does not permit standardization of the viral inoculum (20, 21), and polymerase chain reaction-based assays directed at specific regions of the viral reverse transcriptase (RT) are dependent upon prior knowledge of the mutations important in antiviral resistance (2, 13) and are not applicable to agents which inhibit human immunodeficiency virus (HIV) by mechanisms other than RT inhibition.

To provide an assay that would support the growth of most if not all clinical isolates, permit standardization of the viral inoculum in the same cell system used for the assay, and be applicable to antiretroviral agents with differing mechanisms of action, we have developed a microtiter, peripheral blood mononuclear leukocyte (PBL)-based assay system. In this report, we describe this replication endpoint concentration assay and illustrate its utility by applying it to the determination of the dideoxynucleoside susceptibility profiles of pre- and posttherapy isolates derived from patients entered into a phase I trial of DDI.

MATERIALS AND METHODS

Patients. Thirty patients were enrolled in a phase I study of DDI at the New England Deaconess Hospital in July 1989. Viral isolates from 13 patients who remained on DDI therapy consistently for more than 5 months were studied for their dideoxynucleoside susceptibility profile. Ten patients had AIDS, and three had AIDS-related complex. The mean CD4 count was 84/mm^3 (range, 10 to 310/mm^3). Before entry into the study, all of the patients had been on AZT (mean duration, 6.5 months; range, 1 to 13 months). DDI was administered at a dose of 750 mg/day. The clinical results of this trial have been reported separately (7).

Virus isolation and titer determination. Virus was isolated from the patients before starting DDI therapy, and a second

* Corresponding author.

isolate was obtained when the patients had been on therapy for a mean of 57.8 weeks (range, 21 to 90 weeks). The clinical isolates were obtained by isolation of PBLs by using a Ficoll-Isopaque gradient (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.). The PBLs (5×10^6) were cocultivated with 5×10^6 HIV-1-negative donor PBLs that were prestimulated with 3 μg of phytohemagglutinin (PHA; Sigma, St. Louis, Mo.) per ml. The cells were maintained in RPMI 1640 medium (GIBCO, St. Louis, Mo.) supplemented with 5% interleukin-2 (Pharmacia, Columbia, Md.), 20% fetal bovine serum, 250 U of penicillin per ml, 250 μg of streptomycin per ml, 2 mM L-glutamine, and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer. The medium was changed every 3 to 4 days, and 5×10^6 PHA-stimulated PBLs were added every week. The cell-free supernatant was removed and tested for HIV p24 antigen by enzyme immunoassay (Abbott Laboratories, Abbott Park, Ill.). The cell-free supernatant from positive cultures was aliquoted and frozen at −70°C. Clinical isolates were never passaged in PBLs more than twice. One laboratory strain, IIIB (courtesy of R. Gallo, National Institutes of Health, Bethesda, Md.), and one clinical isolate, 906, obtained from a patient who had never received AZT or DDI therapy, were used as control strains. In addition, two AZT-susceptible strains, A012-pre and A018-pre, and two AZT-resistant strains, A012-post and A018-post, were obtained from the AIDS Research and Reference Reagent Program, Rockville, Md., and also used as control strains in all experiments (12).

To determine the titer of each viral strain, a 50% tissue culture infectious dose in PBLs ($TCID_{50}$) was determined. A 20-μl volume of supernatant was serially diluted 10-fold in 96-well plates; six separate dilutions per strain were performed on each plate, leaving the outer wells empty. Then 10^5 PHA-stimulated donor PBLs were added to each well. The cells were incubated at 37°C for 1 week without changing the media. At 1 week the supernatant of each well was removed and the HIV p24 antigen level was measured. Each well that contained more than 30 pg of p24 antigen per ml was considered positive. The $TCID_{50}$ was calculated by using the method of Reed and Muench (16).

Replication endpoint concentration assay. A $TCID_{50}$ of 10^5 was used to infect 10^7 PHA-stimulated PBLs. The cells were suspended for 1 h at 37°C in the viral inoculum in a total volume of 1 ml. They were washed twice and centrifuged at 2,000 rpm ($833 \times g$) and the supernatant was removed and discarded. Then 10^5 PBLs were placed in 200 μl of medium containing DDI (courtesy of H. Mitsuya, National Institutes of Health, Bethesda, Md.) or AZT (Burroughs-Wellcome, Research Triangle Park, N.C.). The concentrations of DDI tested were 1, 2.5, 5, 10, 20, 25, and 50 μM, and those of AZT were 0.01, 0.1, 1, 10, and 100 μM. All strains were tested in duplicate. The supernatant (100 μl) was removed at 1 week and diluted with 100 μl of medium, and the amount of p24 antigen was measured. Because of the dilution, the amount of p24 antigen was doubled when determining replication assay results. The replication endpoint concentration was the highest drug concentration at which >1,000 pg of antigen per ml was detected in both wells. If one of the two wells had a p24 antigen level of >1,000 pg/ml and the other well had a p24 antigen level of <500 pg/ml, the average of the drug concentrations in those wells and the next lowest drug concentration was determined to be the replication endpoint concentration. If one of the two wells had a p24 antigen level of >1,000 pg/ml and the other well had a p24 antigen level of >500 pg/ml, the replication endpoint concentration was determined to be the drug concentration in those wells. The level of 1,000 pg/ml was chosen as the endpoint for this assay because it reflects the accurate upper-end measurement of the standard curve for the Abbott enzyme immunoassay (500 pg/ml).

To correlate the replication endpoint concentration with a formal percent inhibitory concentration, we reassayed eight strains and obtained the absolute p24 antigen content for each drug concentration. The concentrations of drug that reduced the p24 antigen value of the control well by 50% (IC_{50}) and by 90% (IC_{90}) were calculated by using nonparametric regression analysis. This analysis employed the median-effect equation, $f_a = 1/[1 + (D_m/D)]^m$, where D is the dose, f_a is the fraction affected, and D_m is the dose required to produce the median effect (6), and was carried out by using software from Systat Inc., Evanston, Ill. By entering the doses of drug used and the percent reduction in the amount of HIV p24 antigen (i.e., the fraction affected), we calculated the median dose. The solved equation was then put into graphic form by using Excel software (Microsoft Corp., Redmond, Wash.).

Plasma viral cultures. Plasma obtained from patients was filtered through a 0.45-μm syringe filter. Then 2 ml of the filtered plasma was added to 10^7 PHA-stimulated, HIV-negative PBLs and incubated at 37°C for 1 h. The suspended cells in plasma were then added to 7.5 ml of medium plus 0.5 ml of interleukin-2 and placed in a 25-cm^2 flask. The medium was changed every 3 to 4 days, and 5×10^6 PBLs were added every week. The supernatant was assayed twice per week, and the culture was considered positive if the supernatant p24 antigen concentration was greater than 1,000 pg/ml or increased fourfold from the previous sample.

Statistics. The Wilcoxon signed-rank test was used to analyze the changes in susceptibility to AZT and DDI over time. The two-sampled t test was used to analyze initial DDI susceptibility levels in relation to surrogate markers such as the CD4 cell count. In analyzing the susceptibility to DDI in relation to the change in CD4 count, we chose 2.5 μM as the cutoff because of a report indicating that HIV-1 isolates not previously exposed to DDI have 50% inhibitory doses (ID_{50}s) of <2.5 μM (26).

RESULTS

The replication endpoint concentration assay was successful in determining the dideoxynucleoside susceptibilities of every isolate that was cultured in PBLs. One patient (patient 3) became culture negative after receiving DDI and has remained so through 90 weeks of therapy; therefore, only his pretherapy isolate was available for testing. The isolates from the other patients and the control strains were all tested for AZT and DDI sensitivity by using the 1-week endpoint concentration assay. Eight isolates were tested by using a 2-week endpoint concentration assay that involved changing 100 μl of medium after 1 week. The 2-week assay revealed endpoint concentrations equal to or one concentration higher than the 1-week values (data not shown). When absolute values of p24 antigen levels were determined, the replication endpoint concentration corresponded to a 90 to 99% reduction in p24 antigen levels when compared with control wells (Fig. 1). Thus, this replication endpoint concentration assay is equivalent to a 90 to 99% inhibitory concentration (IC_{90-99}). The reproducibility of this assay is reflected by the fact that, in analyzing 196 replicates of the assay examining 31 HIV-1 strains, the replication endpoint concentration was within one dilution 97.4% of the time.

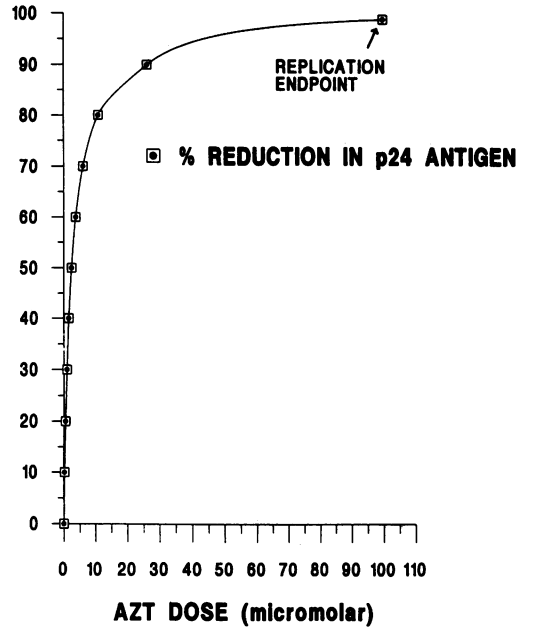

FIG. 1. The AZT inhibitory concentration (IC) curve for the pretherapy isolate from patient 8. The replication endpoint concentration for this isolate corresponds to an IC_{97}.

The assay revealed that for the six control strains the median replication endpoint concentration was 1.8 μM for DDI (mean, 3.4 μM; range, 1 to 10 μM) (Table 1). The median and mean replication endpoint concentrations of AZT were both 0.033 μM (range, 0.01 to 0.055 μM) for the four control strains that had not been previously exposed to AZT. For both strains 012-post and 018-post (resistant controls) the concentrations were greater than or equal to 100 μM. For the 13 baseline clinical isolates, the median endpoint concentration of AZT was 1.0 μM, with a mean of 25.6 μM and a range of 0.01 to 100 μM (Table 2). While the patients were off AZT and receiving DDI, the median endpoint concentration of AZT for the posttherapy isolates was 0.3 μM, with a mean of 10.9 μM and a range of 0.01 to 100 μM. The AZT endpoint concentrations decreased for 9 of the 12 paired isolates while the patients were off AZT therapy, while the values for the other 3 pairs remained unchanged. The improvements in susceptibility to AZT ranged from 2- to 1,000-fold ($P = 0.008$; Wilcoxon signed-rank test).

TABLE 1. Replication endpoint concentrations for control strains

Strain	Replication endpoint concn (μM) of:	
	AZT	DDI
AZT susceptible		
906	0.055	10
IIIB	0.01	5
A012-pre	0.01	1
A018-pre	0.055	1
Median	0.033	3.0
Mean	0.033	4.3
AZT resistant		
A012-post	≥100	2.5
A018-post	≥100	1
Median	≥100	1.8
Mean	≥100	1.8

TABLE 2. AZT replication endpoint concentrations

Patient	Weeks of DDI	Replication endpoint concn (μM) of AZT:	
		Before DDI therapy	After DDI therapy[a]
1	80	10	10
2	24	0.1	0.055
3	90	0.055	NEG[b]
4	21	10	0.1
5	51	1	0.55
6	73	10	0.01
7	50	0.1	0.01
8	83	100	100
9	53	100	10
10	52	1	0.1
11	70	0.1	0.01
12	54	100	10
13	50	0.01	0.01
Median	53.0	1.0[a]	0.3[a]
Mean	57.8	25.6	10.9
SD	20.2	41.0	27.2

[a] $P = 0.008$ between pre- and posttherapy values (Wilcoxon test).
[b] NEG, negative.

The median endpoint concentration of DDI for the 13 clinical isolates prior to DDI therapy was 10.0 μM, with a mean of 9.5 μM and a range of 1 to 25 μM (Table 3). Following a mean period of 57.8 weeks, the median endpoint concentration of DDI for the isolates was 17.5 μM, with a mean of 16.7 μM and a range of 2.5 to 50 μM. The DDI replication endpoint concentrations increased for 9 of the 12 paired isolates while the patients were on therapy. The increases in DDI endpoint concentrations ranged from 50% to threefold ($P = 0.036$; Wilcoxon signed-rank test).

A retrospective analysis of surrogate markers obtained during the first 12 weeks of the study was done (Table 3). These markers included CD4 count, p24 antigen level in plasma, and plasma viral culture. Most notably, the patient (patient 3 in Table 3) with the lowest replication endpoint concentration (1 μM) became PBL culture negative. Further analysis of these data is limited by the small number of patients. However, the three patients (patient 3, 6, and 13 in Table 3) with the lowest initial DDI replication endpoint concentrations (1 to 2.5 μM) had the greatest increase in CD4 counts (mean increase, 88/mm^3). The remaining 10 patients with replication endpoint concentrations of >2.5 μM had a mean increase of only 19/mm^3 in their CD4 counts ($P = 0.02$; t test). A rank correlation test revealed that patients with the highest initial CD4 counts had the greatest increase in CD4 count while on therapy ($P = 0.035$). Other trends which were observed but which did not reach statistical significance were as follows: (i) the patients with CD4 counts of <50/mm^3 were more likely to have plasma viremia (50 versus 28.6%); (ii) the patients whose DDI replication endpoint concentrations increased while on therapy had lower initial CD4 counts than the patients whose DDI replication endpoint concentrations decreased or remained unchanged (58 versus 126/mm^3); and (iii) the patients whose DDI replication endpoint concentrations increased were also more likely to be p24 antigenemic than the patients whose

TABLE 3. DDI replication endpoint concentrations and surrogate markers

Patient	Weeks of DDI	Replication endpoint concn (μM) of DDI:		Initial CD4 count	Change in CD4 count	Initial p24 antigen	Plasma viral culture[a]
		Before therapy	After therapy				
1	80	5	15	40	−18	65	ND
2	24	5	5	60	−20	0	ND
3	90	1	NEG	234	66	0	NEG
4	21	10	5	18	12	0	NEG
5	51	7.5	10	60	40	70	NEG
6	73	2.5	5	80	110	15	NEG
7	50	10	20	20	10	17	POS
8	83	25	50	10	10	0	NEG
9	53	25	37.5	60	30	0	NEG
10	52	10	5	10	30	10	POS
11	70	10	20	66	64	115	POS
12	54	10	25	130	30	51	POS
13	50	2.5	2.5	310	90	0	NEG
Median	53.5	10.0[b]	17.5[b]	60.0	30.0	12.5	
Mean	57.8	9.5	16.7	84.5	34.9	26.4	
SD	20.2	7.3	14.2	87.3	37.4	35.6	

[a] POS, positive; NEG, negative; ND, not done.
[b] $P = 0.036$ between pre- and posttherapy values (Wilcoxon test).

DDI replication endpoint concentrations decreased or remained unchanged (75 versus 33%). No correlation was noted between in vitro susceptibilities and changes in serum HIV p24 antigen levels.

DISCUSSION

In this report we have described a microtiter, PBL-based assay system which can be used both to screen and to directly compare low-passage isolates of HIV-1 for their antiviral susceptibility profiles. Its advantages are that it is easy to perform, the viral inocula are standardized in the same cell system (i.e., PHA-stimulated PBLs) as the antiviral assay, all clinical strains cultured on PBLs can be assayed, results are available relatively quickly for a culture-based system, it is applicable to antiviral agents with differing mechanisms of action, and the endpoint is clear-cut. The supernatant HIV p24 antigen concentration is used to determine the endpoint, given the sensitivity and availability of the commercial enzyme immunoassay kits. To minimize cost, the endpoint is determined as the microtiter well with the highest concentration of drug which exhibits an off-scale reading (i.e., an optical density of >2.0 in our assay). Dilutions of supernatant to determine specific HIV p24 antigen values are not necessary, thus saving substantial technical time and cost.

It is important to note that by using this replication endpoint concentration assay to determine the susceptibility of an isolate, the values provided are IC_{90-99}s. Most previous studies have determined IC_{50}s (2, 9, 11–14, 18–20), but in our assay a higher value (IC_{90-99}) is determined. The inhibition of viral replication by dideoxynucleosides is dependent on the cell type used in the assay because of different rates of phosphorylation in cells. Furthermore, the assay systems reported to date have used different viral inocula and various determinations of HIV-1 activity including plaque reduction, RNA-RNA hybridization, RT activity, and p24 antigen level in supernatants. Thus, differences from previously published values for particular isolates may result from differences in assay systems, from our use of a high inoculum (10^5 $TCID_{50}$s/10^7 PBLs), or because IC_{90-99}s rather than IC_{50}s are reported.

When applied to a number of control strains, the replication endpoint concentration assay clearly distinguished between AZT-susceptible and AZT-resistant isolates and yielded DDI replication endpoint concentrations in a susceptibility range comparable to that of prior reports for these previously DDI-unexposed strains (Table 1) (9, 11). When applied to the 13 pairs of clinical isolates derived from patients in a phase I DDI trial (7), the replication endpoint concentration assay yielded a number of interesting observations. First, its ready applicability to low-passaged clinical isolates was evident. Second, median baseline (pre-DDI) susceptibilities to AZT were 1.0 μM, with a mean of 25.6 μM and a range of 0.01 to 100 μM. This overall diminished susceptibility to AZT is in keeping with the nature of the patient population from whom the isolates were derived; that is, all individuals had advanced HIV disease and were uniformly exposed to AZT (10, 12, 13, 21). Interestingly, with time off AZT and on DDI, AZT susceptibilities improved. This finding is in accord with the results of Bach et al. (1) and St. Clair et al. (26), who reported improvements in mean AZT susceptibility from 13 to 5 μM and >10 to 0.73 μM, respectively, in clinical isolates from patients off AZT. This improvement has been attributed to a Leu-to-Val mutation at position 74 in the viral RT, which has been linked to diminished DDI susceptibility and appears to simultaneously confer improved AZT susceptibility when at least one of the previously described AZT resistance mutations is present (at position 67, 70, 215, or 219) (14, 15, 26). This improvement in AZT susceptibility of isolates from patients off AZT therapy may not be a uniform occurrence, as illustrated by two of our isolate pairs (from patients 1 and 8, Table 2) and in a previously published report (24). Nevertheless, the potential for improvement in AZT susceptibility while off therapy supports treatment strategies that involve the concept of drug cycling to maximize the effectiveness of the dideoxynucleosides.

A third observation was that baseline (pretherapy) DDI replication endpoint concentrations varied 25-fold. This

broad range of susceptibilities to DDI may be more evident than in previous reports because these values represent at least the IC_{90}s of these isolates, as noted above. Nevertheless, a broader range of baseline DDI susceptibilities of previously DDI-unexposed isolates contrasts with baseline AZT susceptibilities of previously AZT-unexposed isolates, which generally are found to fall in a narrow range (Table 1) (9, 12, 14, 18). Following a mean period of 57.8 weeks on DDI therapy, a significant trend toward diminished susceptibility to DDI was seen (Table 3). Two- to three-fold decreases in susceptibility were noted for a number of the isolates, but in no case was the >100-fold decrease in susceptibility found that has been reported for AZT-resistant isolates (9, 11, 12, 14, 18). Similar findings have recently been reported by other investigators. Using a PBL-based assay and testing RT activity, St. Clair et al. reported that the mean ID_{50} of DDI increased from 0.3 to 9.0 μM after 12 months of therapy (26). Reichman et al., using a PBL- and HIV p24 antigen-based assay, reported that MICs of DDI increased from 5 to 10 μM in patients on therapy for 1 year (17). In the recent report by St. Clair et al. (26), the DDI resistance was linked to the Leu-to-Val substitution at position 74 in the HIV RT as noted above. Overall, the contrasting patterns of resistance seen with AZT and DDI therapy indicate that one cannot extrapolate from the experience with one nucleoside analog to another, that exposure to one drug may influence the susceptibility to another agent, and that this influence may result in divergent susceptibility patterns (17, 26).

Baseline DDI susceptibility and CD4 counts at the initiation of treatment may be useful as predictors of response to DDI therapy. In our small cohort, the subject with the most susceptible viral isolate became reproducibly culture negative. Furthermore, rises in CD4 counts were significantly correlated with both initially greater DDI susceptibility and higher baseline CD4 counts. The correlation of higher baseline CD4 counts with subsequent CD4 increases after DDI treatment has also been seen in other trials (3, 22). Isolates that exhibited a rise in replication endpoint concentrations (i.e., a diminished susceptibility) were more likely to be derived from patients who were plasma culture positive or HIV p24 antigenemic at baseline. Although these trends were not statistically significant, they are in keeping with the concept that a greater viral load is more likely to lead to the development of resistance with prolonged therapy. Because the viral load has been clearly correlated with the stage of disease, the predictable development of resistance in patients with advanced HIV disease compared with individuals with asymptomatic or early symptomatic disease supports the hypothesis that viral load is a risk factor for dideoxynucleoside resistance evolving with drug exposure (8, 23).

The correlation of in vitro dideoxynucleoside resistance with clinical events has not been firmly established but is suggested by two recent reports. In the Canadian Multi-Centre AZT trial, patients with CD4 counts of >270 whose viruses developed resistance had a greater than expected progression to AIDS or AIDS-related complex (25). A second study, involving 18 children, noted a significant correlation between decreased AZT susceptibility and poor clinical outcome (27). A number of studies are now under way or are planned to try to secure the correlation of dideoxynucleoside resistance with poorer clinical outcome and to determine whether dideoxynucleoside susceptibility profiles are useful in guiding therapeutic decisions. Assays such as the one described herein should assist in this important effort.

ACKNOWLEDGMENTS

S.M.H. is supported by Public Health Service grants AI27659 and HL43510 from the National Institutes of Health and by Defense Department contract DAMD-17-90-C-0106.

REFERENCES

1. **Bach, M. C., M. St. Clair, D. King, and C. Vavro.** 1991. Long-term follow up of patients with HIV isolates resistant to zidovudine (AZT) treated with dideoxyinosine (DDI). Abstr. no. TU.B.91, p. 80. Abstr. VII Int. Conf. AIDS. 1991.
2. **Boucher, C. A. B., M. Tersmette, M. A. Lange, P. Kellam, R. E. Y. DeGoede, J. W. Mulder, G. Darby, J. Goudsmit, and B. A. Larder.** 1990. Zidovudine sensitivity of human immunodeficiency viruses from high-risk, symptom-free individuals during therapy. Lancet **336**:585–590.
3. **Butler, K. M., R. N. Husson, F. M. Balis, P. Brouwers, J. Eddy, D. El-Amin, J. Gress, M. Hawkins, P. Jarosinski, H. Moss, D. Poplack, S. Santacroce, D. Venzon, L. Wiener, P. Wolters, and P. A. Pizzo.** 1991. Dideoxyinosine in children with symptomatic human immunodeficiency virus infection. N. Engl. J. Med. **324**:137–144.
4. **Chesebro, B., and K. Wehrly.** 1988. Development of a sensitive quantitative focal assay for human immunodeficiency virus infectivity. J. Virol. **62**:3779–3788.
5. **Chesebro, B., K. Wehrly, J. Metcalf, and D. E. Griffin.** 1991. Use of a new CD4-positive HeLa cell clone for direct quantitation of infectious human immunodeficiency virus from blood cells of AIDS patients. J. Infect. Dis. **163**:64–70.
6. **Chou, T. C., and P. Talalay.** 1986. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Regul. **22**:27–55.
7. **Connolly, K. J., J. D. Allan, H. Fitch, L. Jackson-Pope, C. McLaren, R. Canetta, and J. E. Groopman.** 1991. Phase I study of 2′-3′-dideoxyinosine administered orally twice daily to patients with AIDS or AIDS-related complex and hematologic intolerance to zidovudine. Am. J. Med. **91**:471–478.
8. **Ho, D. D., T. Moudgil, and M. Alam.** 1989. Quantitation of human immunodeficiency virus type 1 in the blood of infected persons. N. Engl. J. Med. **321**:1621–1625.
9. **Japour, A. J., P. A. Chatis, H. A. Eigenrauch, and C. S. Crumpacker.** 1991. Detection of human immunodeficiency virus type 1 clinical isolates with reduced sensitivity to zidovudine and dideoxyinosine by RNA · RNA hybridization. Proc. Natl. Acad. Sci. USA **88**:3092–3096.
10. **Land, S., G. Treloar, D. McPhee, C. Birch, R. Doherty, D. Cooper, and I. Gust.** 1990. Decreased in vitro susceptibility to zidovudine of HIV isolates obtained from patients with AIDS. J. Infect. Dis. **161**:326–329.
11. **Larder, B. A., B. Chesebro, and D. D. Richman.** 1990. Susceptibilities of zidovudine-susceptible and -resistant human immunodeficiency virus isolates to antiviral agents determined by using a quantitative plaque reduction assay. Antimicrob. Agents Chemother. **34**:436–441.
12. **Larder, B. A., G. Darby, and D. D. Richman.** 1989. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science **243**:1731–1734.
13. **Larder, B. A., P. Kellam, and S. D. Kemp.** 1991. Zidovudine resistance predicted by direct detection of mutations in DNA from HIV-infected lymphocytes. AIDS **5**:137–144.
14. **Larder, B. A., and S. D. Kemp.** 1989. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). Science **246**:1155–1158.
15. **Mayers, D., F. E. McCutchan, E. E. Sanders-Buell, L. I. Merritt, A. K. Fowler, N. M. Ruiz, and D. S. Burke.** 1991. Characterization of HIV-1 isolates arising after prolonged zidovudine therapy. Abstr. no. W.B.2118, p. 211. Abstr. VII Int. Conf. AIDS. 1991.
16. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27**:493–497.
17. **Reichman, R., J. Lambert, J. Strussenberg, and R. Dolin.** 1991. Decreased dideoxyinosine (DDI) sensitivity of HIV isolates obtained from long term recipients of DDI. Abstr. no. TU.B.92, p. 80. Abstr. VII Int. Conf. AIDS. 1991.

18. **Richman, D. D.** 1990. Susceptibility to nucleoside analogues of zidovudine-resistant isolates of human immunodeficiency virus. Am. J. Med. **88:**8S–10S.
19. **Richman, D. D., J. M. Grimes, and S. W. Lagakos.** 1990. Effect of stage of disease and drug dose on zidovudine susceptibilities of isolates of human immunodeficiency virus. J. Acquired Immune Defic. Syndr. **3:**743–746.
20. **Rooke, R., M. A. Parniak, M. Tremblay, H. Soudeyns, X. Li, Q. Gao, X.-J. Yao, and M. A. Wainberg.** 1991. Biological comparison of wild-type and zidovudine-resistant isolates of human immunodeficiency virus type 1 from the same subjects: susceptibility and resistance to other drugs. Antimicrob. Agents Chemother. **35:**988–991.
21. **Rooke, R., M. Tremblay, H. Soudeyns, L. DeStephano, X.-J. Yao, M. Fanning, J. S. G. Montaner, M. O'Shaughnessy, K. Gelmon, C. Tsoukas, J. Gill, J. Ruedy, M. A. Wainberg, and the Canadian Zidovudine Multi-Centre Study Group.** 1989. Isolation of drug-resistant variants of HIV-1 from patients on long-term zidovudine therapy. AIDS **3:**411–415.
22. **Rozencweig, M., C. McLaren, M. Beltangady, J. Ritter, R. Canetta, L. Schacter, S. Kelley, C. Nicaise, L. Smaldone, L. Dunkle, R. Barbhaiya, C. Knupp, A. Cross, M. Tsianco, and R. R. Martin.** 1990. Overview of Phase I trials of 2′,3′-dideoxyinosine (ddI) conducted on adult patients. Rev. Infect. Dis. **12:**S570–S575.
23. **Saag, M. S., M. J. Crain, W. D. Decker, S. Campbell-Hill, S. Robinson, W. E. Brown, M. Leuther, R. J. Whitley, B. H. Hahn, and G. M. Shaw.** 1991. High-level viremia in adults and children infected with human immunodeficiency virus: relation to disease stage and CD4+ lymphocyte levels. J. Infect. Dis. **164:**72–80.
24. **Shiraska, T., R. Yarchoan, R. Husson, T. Shimada, K. M. Wyvill, S. Broder, and H. Mitsuya.** 1991. HIV may develop resistance preferentially to azidothymidine (AZT) as compared to dideoxycytidine and dideoxyinosine in patients receiving antiviral therapy. Abstr. no. W.A.9, p. 24. Abstr. VII Int. Conf. AIDS. 1991.
25. **Singer, J., J. S. G. Montaner, M. T. Schechter, T. N. Le, T. Dai, M. Fanning, C. Tsoukas, M. V. O'Shaughnessy, J. Ruedy, and M. Wainberg.** 1991. Clinical significance of in-vitro HIV resistance to zidovudine in early HIV infected individuals after 4 years of therapy. Abstr. no. W.B.2414, p. 285. Abstr. VII Int. Conf. AIDS. 1991.
26. **St. Clair, M. H., J. L. Martin, G. Tudor-Williams, M. C. Bach, C. L. Vavro, D. M. King, P. Kellam, S. D. Kemp, and B. A. Larder.** 1991. Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1. Science **253:**1557–1559.
27. **Tudor-Williams, G., M. H. St. Clair, R. E. McKinney, M. Maha, E. Walter, S. Santacroce, M. Mintz, K. O'Donnell, T. Rudoll, C. L. Vavro, E. M. Connor, and C. M. Wilfert.** 1992. HIV-1 sensitivity to zidovudine and clinical outcome in children. Lancet **339:**15–19.