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Dideoxynucleoside Resistance Emerges with Prolonged Zidovudine Monotherapy

DOUGLAS L. MAYERS,[1]* ANTHONY J. JAPOUR,[2] JEAN-MARIE ARDUINO,[3] SCOTT M. HAMMER,[4] RICHARD REICHMAN,[5] KENNETH F. WAGNER,[1] RAYMOND CHUNG,[1] JAMES LANE,[1] CLYDE S. CRUMPACKER,[2] GAVIN X. McLEOD,[4] LAUREL A. BECKETT,[3] CHESTER R. ROBERTS,[1] DEAN WINSLOW,[6] DONALD BURKE,[1] AND THE RV43 STUDY GROUP[1]†

Military Medical Consortium for Applied Retroviral Research, Rockville, Maryland 20850[1]; Division of Infectious Diseases, Beth Israel Hospital,[2] and Division of Infectious Diseases, New England Deaconess Hospital,[4] Harvard Medical School, and Statistical Analysis and Data Center, Harvard School of Public Health,[3] Boston, Massachusetts 02215; Infectious Disease Unit, University of Rochester Medical Center, Rochester, New York 14642[5]; and DuPont-Merck Pharmaceutical Company, Wilmington, Delaware 19880[6]

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Human immunodeficiency virus type 1 (HIV-1) isolates resistant to zidovudine (ZDV) have previously been demonstrated to exhibit in vitro cross-resistance to other similar dideoxynucleoside agents which contain a 3′-azido group. However, cross-resistance to didanosine (ddI) or dideoxycytidine (ddC) has been less well documented. ZDV, ddI, and ddC susceptibility data have been collected from clinical HIV-1 isolates obtained by five clinical centers and their respective retrovirology laboratories. All subjects were treated only with ZDV. Clinical HIV-1 isolates were isolated, amplified, and assayed for drug susceptibility in standardized cultures of phytohemagglutinin-stimulated donor peripheral blood mononuclear cells obtained from healthy seronegative donors. All five cohorts showed a correlation between decreased in vitro susceptibility to ZDV and decreased susceptibility to ddI and ddC. For each 10-fold decrease in ZDV susceptibility, an average corresponding decrease of 2.2-fold in ddI susceptibility was observed (129 isolates studied; $P < 0.001$, Fisher's test of combined significance). Similarly, susceptibility to ddC decreased 2.0-fold for each 10-fold decrease in ZDV susceptibility (82 isolates studied; $P < 0.001$, Fisher's test of combined significance). These data indicate that a correlation exists between HIV-1 susceptibilities to ZDV and ddI or ddC for clinical HIV-1 isolates.

Zidovudine (ZDV) was the first antiretroviral agent approved for treatment of individuals infected with human immunodeficiency virus type 1 (HIV-1). Shortly after approval by the Food and Drug Administration, ZDV resistance was detected in viruses from individuals treated with ZDV (16, 18, 31). Subsequently, the dideoxynucleoside agents didanosine (ddI) and dideoxycytidine (ddC) were also approved for antiretroviral therapy, and drug-resistant mutant HIV-1 isolates have been detected from patients treated with these agents as well (4, 5, 9, 10, 22, 26, 32).

ZDV, ddI, and ddC are structurally distinct nucleoside analogs; they all function, however, as competitive inhibitors of the HIV-1 reverse transcriptase. The most common precursor to the development of ZDV resistance appears to be the Thr-215→Tyr mutation in the HIV-1 reverse transcriptase; several other mutations, however, have been shown to increase the ZDV 50% inhibitory concentration (IC_{50}) (1, 6, 12, 14, 17, 19–21, 24, 30). The HIV-1 reverse transcriptase mutation Leu-74→Val confers resistance to ddI, while Thr-69→Asp confers resistance to ddC (5, 32). Cross-resistance between ddI and ddC has been shown with mutations at codons for Leu-74→Val and Met-184→Val (7, 9, 32). HIV-1 variants with multiple ZDV resistance mutations combined with Val-74 demonstrated higher ddI resistance than variants with Val-74 alone or Val-74 combined with Tyr-215 (32). HIV-1 isolates have been demonstrated to exhibit in vitro cross-resistance between ZDV and other dideoxynucleoside agents containing a 3′-azido group; cross-resistance between ZDV and ddI or ZDV and ddC, however, has been less well documented (4, 10, 31) and was not observed in some studies (5, 17, 18, 27, 28, 32, 34).

The range of ZDV and ddI susceptibilities by using the AIDS Clinical Trials Group-Department of Defense (ACTG-DoD) consensus drug susceptibility protocol has recently been determined (11). IC_{50}s for ZDV-sensitive HIV-1 isolates range from <0.01 to 0.20 μM, while IC_{50}s for ZDV-resistant HIV-1 isolates are as high as ≥10 μM. Thus, the range of ZDV susceptibilities is greater than 100-fold. Unlike ZDV, the range of ddI susceptibilities is narrow. IC_{50}s for ddI-susceptible isolates range from <0.1 to 4.0 μM, while IC_{50}s for HIV-1 isolates from patients exposed to ddI can be 5.0 to >25.0 μM. Drug levels above 10.0 μM for ZDV and 30.0 μM for ddI are difficult to evaluate on phytohemagglutinin (PHA)-stimulated donor peripheral blood mononuclear cells (PBMC) because of increasing cytotoxicity seen at higher drug levels. The difference in the magnitude of peak IC_{50}s for ZDV and ddI resistance after drug exposure is striking and suggests that a large number of clinical isolates may need to be analyzed to

* Corresponding author. Present address: 13 Taft Ct., Suite 201, Rockville, MD 20850. Phone: (301) 295-6411. Fax: (301) 295-6422. Electronic mail address: maye6832@hjfhiv.hjf.org.

† RV43 Study Group: at National Naval Medical Center, Kenneth Wagner, Nancy Ruiz, Marie Fujimara-Justice, Paul Kernozek, Jane Koch, and Candace Brewer; at Walter Reed Army Medical Center, Raymond Chung, Charles Hicks, M. Elayne Shellie, Li Loriz-Lim, Cyrilla Smalls, and Arthur Hamilton; at Walter Reed Army Institute of Research, Douglas L. Mayers, Donald S. Burke, and Chester R. Roberts; at Henry M. Jackson Foundation Laboratory, Francine McCutchan and Eric Sanders-Buell; and at SRA Technologies, Owen Weislow, Arnold Fowler, James Lane, and Linda Merritt.

TABLE 1. Patient characteristics

Group	Disease stage (no. of patients)				ZDV therapy				CD4 count			
	Total[a]	AIDS	AIDS-related complex	Asymptomatic	Total[a]	Duration (mo)			Total[a]	Cells/mm^3		
						Mean	Median	Range		Mean	Median	Range
MMCARR	66	3	19	44	61	19.0	19	1–53	66	197	172	4–700
BIH	22	9	11	2	22	7.2	5.5	0–24	22	85	66	7–224
DM	15	13	0	2	14	17.9	18	1–36	15	36	11.5	2–259
NEDH	13	10	3	0	13	6.5	5	1–13	13	84	90	10–310
URoch	12	9	3	0	12	9.4	9	2.5–20	12	113	101.5	0–247
Total	128	44	36	48	122	14.7	13	0–53	128	142	93	0–700

[a] Represents total number of clinical isolates in analyses. Information on stage of disease, length of ZDV therapy, or CD4 count was not available for all patients.

establish the relationship between ZDV and ddI or ddC susceptibility.

The objective of this study was to determine whether there is a correlation between the susceptibility of HIV-1 to ZDV and ddI or ddC. Clinical HIV-1 isolates derived from patients who had been treated with ZDV therapy but no previous ddI or ddC therapy were evaluated. Data collected by different laboratories using several PBMC-based drug susceptibility assays were analyzed to increase the likelihood of observing a true biological relationship.

MATERIALS AND METHODS

Medium. R-3 medium (RPMI 1640 medium supplemented with 15 to 20% heat-inactivated fetal bovine serum, 5 to 10% purified human interleukin-2, 250 U of penicillin per ml, 250 μg of streptomycin per ml, and 2 mM L-glutamine) was used for these studies.

Cells. Donor PBMC were prepared from Ficoll-Hypaque (Histopaque 1077; Sigma) density gradient centrifugation from HIV-1-seronegative donors. PBMC (2×10^6/ml) were stimulated with 2 to 3 μg of PHA P in R-3 medium for 1 to 3 days.

Viral isolation. All clinical HIV-1 isolates were obtained by cocultivation of PHA-stimulated donor PBMC with fresh patient PBMC obtained by Ficoll-Hypaque separation of heparinized blood. Isolates were expanded on fresh donor PBMC to obtain a virus stock which was frozen in aliquots at −70°C (Beth Israel Hospital [BIH], DuPont-Merck Pharmaceutical Co. [DM], New England Deaconess Hospital [NEDH], and University of Rochester Medical Center [URoch]) or −180°C (Military Medical Consortium for Applied Retroviral Research [MMCARR]).

Drug susceptibility assays. (i) Standardized ACTG-DoD HIV-1 drug susceptibility assay (MMCARR, BIH, and DM). The virus stock supernatant titer was determined by endpoint dilution using serial fourfold dilutions as previously described (11). A standardized inoculum (1,000 50% tissue culture infective doses [$TCID_{50}$]/10^6 PBMC) of virus supernatant and PBMC was incubated for 1 h, then unabsorbed virus was removed, and infected PBMC were incubated with various concentrations of ZDV or ddI for 7 days. The ACTG-DoD consensus drug susceptibility assay (MMCARR, BIH, and DM) was modified to include testing of ddI at concentrations of 0.1, 1.0, 5.0, 10.0, and 25.0 μM and ddC at concentrations of 0.01, 0.1, 1.0, and 5.0 μM. The IC_{50} of each drug was determined by comparing the p24 antigen values in the no-drug control wells with the values in the drug-containing wells by using the median-effect equation (3).

(ii) Replication endpoint concentration assay (NEDH). Supernatant was serially diluted by 10-fold dilutions. The $TCID_{50}$ for each viral stock was calculated by the method of Reed and Muench (25). A standardized inoculum (10^4 $TCID_{50}$/10^6 PBMC) of virus supernatant was added to duplicate wells containing medium with ZDV or ddI. The replication endpoint was the highest concentration of drug which contained >1,000 pg of p24 antigen per ml in both wells as previously described (22).

(iii) URoch susceptibility assay. Titers of viral stocks were determined by using triplicate serial 10-fold dilutions added to 1.5×10^6 PHA-stimulated donor PBMC in each well. The $TCID_{50}$ for each viral isolate was calculated by the method of Reed and Muench (25). In the susceptibility assay, each well was inoculated with 50 to 100 $TCID_{50}$ of the HIV-1 isolate to be tested (approximately 30 $TCID_{50}$/10^6 cells). ZDV and ddI were added to the wells to achieve concentrations of 0.01, 0.1, 1.0, and 10 μM ZDV and 5, 10, 50, and 100 μM ddI, respectively. On day 12, virus supernatant was harvested. Supernatants were assayed for p24 antigen by enzyme-linked immunosorbent assay (Abbott). The MIC was defined as the drug concentration at which the p24 antigen in the drug wells was reduced by at least 90% compared with the untreated virus control wells as previously described (26).

Statistics. The drug susceptibilities for each laboratory (IC_{50}s, IC_{90}s, or IC_{95}s) were $\log_{10}$ transformed to achieve normality, and the susceptibilities of the HIV-1 isolates to ZDV and ddI or ddC were evaluated by simple linear regression with calculation of Pearson correlation coefficients and *P* values. The change in ddI or ddC susceptibility with a 10-fold change in ZDV susceptibility was calculated with 95% confidence intervals. Susceptibility results from different laboratories were evaluated by using Fisher's test of combined significance.

RESULTS

Study population. Characteristics of patients whose HIV-1 isolates were assayed in this study are summarized in Table 1. The HIV-1 isolates were evenly distributed among the spectrum of HIV-1 disease stage; 44 of the patients had AIDS, 36 had AIDS-related complex, and 48 had asymptomatic HIV-1 infection. The mean duration of ZDV monotherapy was 14.7 months (range, 0 to 53 months). None of the patients received previous ddI or ddC therapy.

Individual laboratory drug susceptibility assay results. The drug susceptibility assays used by the five laboratories are summarized in Table 2, and results obtained with reference HIV-1 strains are listed in Table 3. The average ZDV IC_{50}s for reference sensitive and resistant HIV-1 isolates by using the ACTG-DoD protocol were 0.02 and 3.5 μM, respectively. The endpoint replication concentration assay (NEDH) produced

TABLE 2. Drug susceptibility assay formats

Group	Assay method	Inoculum ($TCID_{50}$/10^6 PBMC	Concn range (μM) of:			Assay termination (day)	Measurement
			ZDV	ddI	ddC[a]		
MMCARR	ACTG-DoD	1,000	0.001–5.0	0.1–25.0	0.01–1.0 (5.0)	7	IC_{50}
BIH	ACTG-DoD	1,000	0.001–5.0	0.1–25.0	—	7	IC_{50}
DM	ACTG-DoD	1,000	0.001–5.0	0.1–25.0	0.01–1.0 (5.0)	7	IC_{50}
NEDH	Replication endpoint assay[b]	10,000	0.01–100.0	1.0–50.0	—	7	IC_{90-99}
URoch	URoch susceptibility	100	0.001–10.0	5.0–100.0	—	12	IC_{90}

[a] Most assays were performed with ddC concentrations ranging from 0.01 to 1.0 μM; the 5 μM concentration was added for selected HIV isolates. —, not done.
[b] Replication endpoint concentration (22).

ZDV IC_{90-99}s of 0.01 to 0.055 and ≥100 μM for ZDV-sensitive and ZDV-resistant reference isolates, respectively. URoch did not evaluate any reference HIV-1 strains with their assay.

The IC_{50}s for the reference HIV-1 isolates ranged from 0.6 to 6.0 μM for ddI and 0.02 to 0.30 μM for ddC by using the ACTG-DoD protocol. The ddI IC_{90-99}s ranged from 1.0 to 5.0 μM by using the endpoint replication concentration assay.

Correlation of ZDV susceptibility with ddI and ddC susceptibility results. There was a significant linear correlation between the log-transformed ZDV and ddI susceptibility data for all five laboratories (data summarized in Fig. 1 and Table 4). The *R* values for this association ranged from 0.50 to 0.84, and the *P* values for all five studies were significant, ranging from 0.02 to 0.0001. The combined significance of all five studies was $P < 0.001$ by using Fisher's test of combined significance.

For each 10-fold increase in ZDV resistance, a corresponding decrease in ddI susceptibility ranged between 1.5- and 3.9-fold. The 95% confidence intervals for all five studies overlap, with an average 2.2-fold decrease in ddI susceptibility for each 10-fold decrease in ZDV susceptibility. Similar results were observed with ddC, with a linear association between log-transformed ZDV and ddC susceptibility values for clinical HIV-1 isolates ($P = 0.0001$) (Fig. 2 and Table 5). ddC susceptibility decreased 2.0-fold for each 10-fold decrease in ZDV susceptibility.

Sequential HIV-1 isolates. Paired HIV-1 isolates from 20 MMCARR patients on ZDV monotherapy who developed ZDV resistance showed a similar trend of decreased susceptibility to ddI and ddC as ZDV resistance emerged. All of the ZDV-resistant HIV-1 isolates showed decreased susceptibility to ddI ($P < 0.001$, paired *t* test) and 18 of 20 ZDV-resistant isolates showed decreased susceptibility to ddC ($P = 0.175$, paired *t* test) compared with those of previous ZDV-sensitive isolates. The decreases in ddI and ddC susceptibility were consistent with the results described for the unpaired isolates described above, although the trend for ddC was not statistically significant.

DISCUSSION

Previous reports have described cross-resistance of ZDV-resistant virus to dideoxynucleoside agents containing a 3′-azido group (17, 18, 28). Our analysis using data from five different laboratories and three different assay methodologies confirms previous observations that there is a correlation between ZDV and ddI (or ddC) susceptibility in clinical HIV-1 isolates (4, 10, 31). The cause for the decrease in drug susceptibilities seen with ddI and ddC as clinical HIV-1 isolates develop ZDV resistance on ZDV monotherapy has not been determined. Although possible, we do not believe that the correlation is an in vitro artifact due to evaluation of viruses with different growth kinetics or harvest of viral isolates past peak viral replication. The levels of p24 antigen in the no-drug wells in the drug assays did not vary significantly in relation to the IC_{50} measured for ZDV or ddI. Previous assays in which p24 antigen levels were measured for clinical HIV-1 isolates at days 4, 7, and 10 showed consistent p24 antigen rises in the no-drug wells from days 4 to 7 with stabilization of levels between days 7 and 10, suggesting that peak virus production occurs around day 7 in the assay format used for the ACTG-DoD protocol (20a). Recent data suggest that ddI is more active in resting PBMC than in PHA-activated cells such as the cells that were used to evaluate drug susceptibility in this paper (8). Alternatively, there may be genetic changes that remain to be determined in PBMC-derived HIV-1 isolates that produce resistance to both ZDV and ddI. Prolonged antiretroviral therapy may also produce a more diverse quasispecies of viral isolates with resistance to dideoxynucleoside agents. Although only a very limited number of HIV-1 isolates have been evaluated (6 to 30 isolates per drug), similar decreases in drug susceptibility with ZDV resistance are seen with U90152, a nonnucleoside reverse transcriptase inhibitor and an XM323, HIV protease inhibitor but not with other agents of the same classes. There is no relationship seen between ZDV resistance and foscarnet susceptibilities in the 11 HIV-1 isolates evaluated to date (20a).

TABLE 3. Drug susceptibilities of reference HIV-1 isolates to ZDV, ddI, and ddC

Reference strains	ZDV (μM) at:				ddI (μM) at:				ddC (μM) at:	
	MMCARR[a]	BIH	DM	NEDH	MMCARR[a]	BIH	DM	NEDH	MMCARR[a]	DM
ZDV sensitive										
A012 (pre)	0.019 ± 0.012	0.02	0.004	0.01	1.07 ± 0.29	1.2	0.8	1.0	0.031 ± 0.036	0.03
A018 (pre)	0.015 ± 0.006	0.02	0.06	0.055	5.96 ± 2.34	2.0	5.9	1.0	0.028 ± 0.014	0.30
3B	ND	0.005	0.005	0.01	ND	2.0	0.6	5.0	ND	0.03
ZDV resistant										
A012 (post)	2.66 ± 1.18	2.3	2.3	≥100	2.85 ± 0.93	2.0	3.3	2.5	0.044 ± 0.019	0.1
A018 (post)	2.31 ± 0.90	>5	3.5	≥100	5.58 ± 4.56	2.5	3.3	1.0	0.019 ± 0.007	0.1

[a] Mean and standard deviation of results from six separate assays. ND, not done.

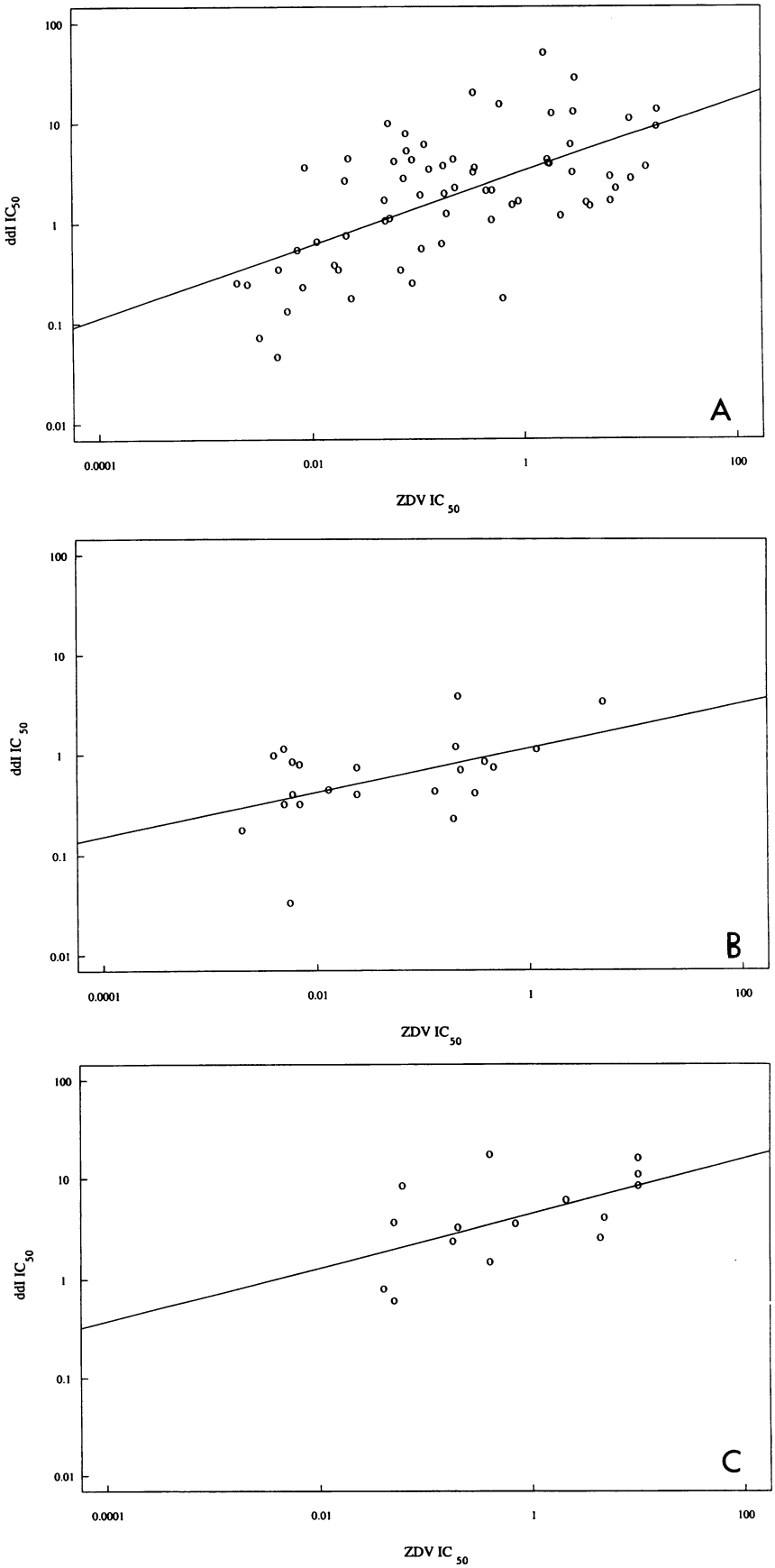
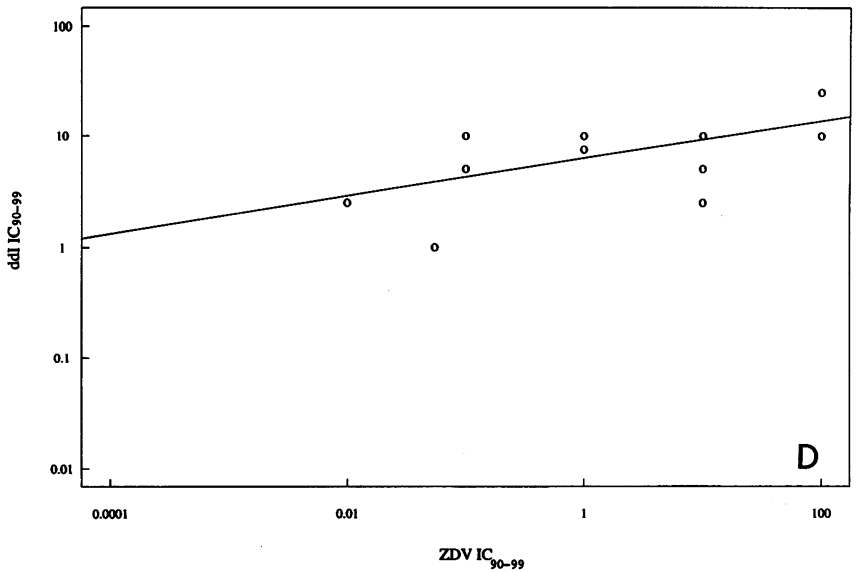
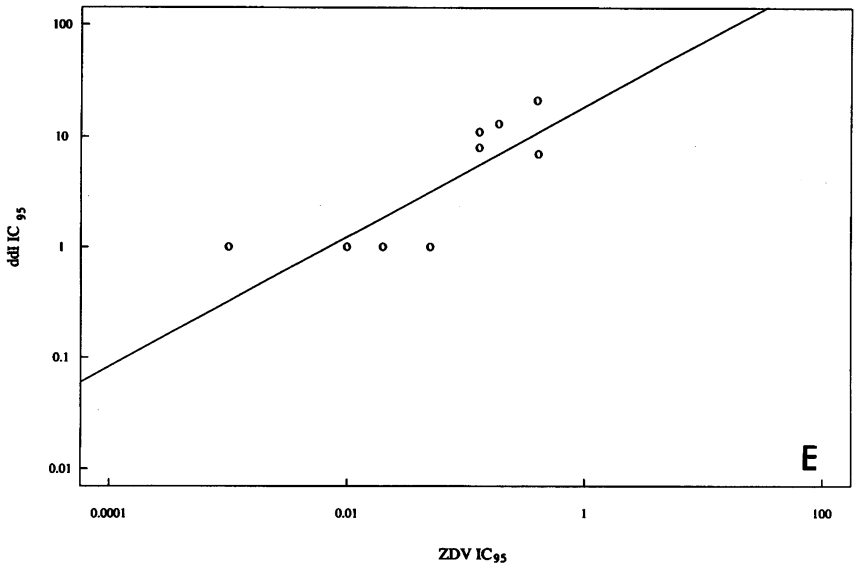


FIG. 1. Comparison of susceptibilities to ZDV and ddI or ddC of HIV-1 isolates from patients on ZDV monotherapy by laboratory. (A) MMCARR; (B) BIH; (C and F) DM; (D) NEDH; (E) URoch. Note the different ranges of IC_{50}s used for ZDV on the *x* axis and ddI or ddC on the *y* axis.





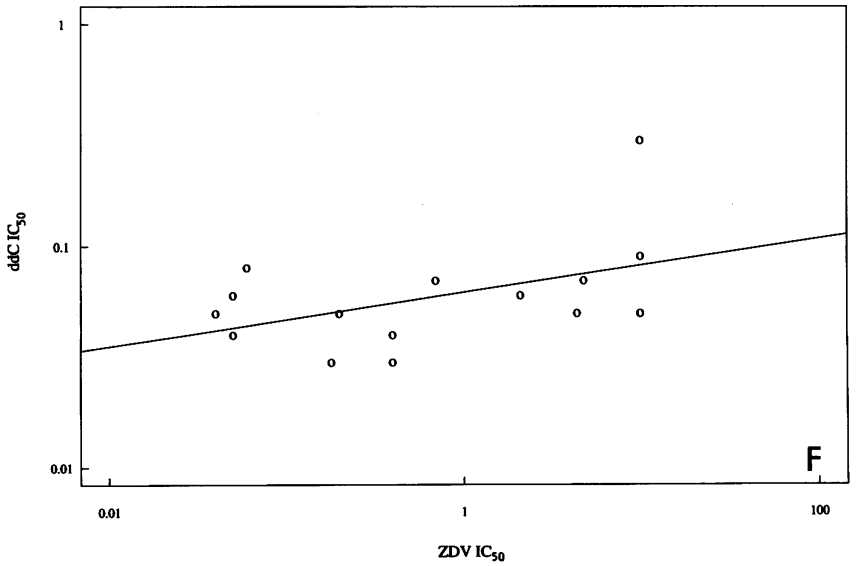


FIG. 1—*Continued.*

TABLE 4. Summary results for susceptibility to ZDV versus susceptibility to ddI by using primary HIV isolates in PBMC-based drug susceptibility assays

Group	N	R	Slope (SE)	Fold change	P value	95% Confidence interval of fold change
MMCARR	67	0.61	0.36 (0.058)	2.3	0.0001	1.8, 3.0
BIH	22	0.50	0.22 (0.086)	1.7	0.02	1.1, 2.5
DM	15	0.57	0.27 (0.11)	1.9	0.03	1.1, 3.2
NEDH	13	0.59	0.17 (0.070)	1.5	0.03	1.0, 2.1
URoch	12	0.84	0.59 (0.12)	3.9	0.0006	2.1, 7.1
Total	129	0.63	0.35 (0.037)	2.2	0.0001	1.9, 2.6

Recent reports from several laboratories suggest that development of ZDV resistance is associated with clinical decline in patients on ZDV monotherapy (15, 23, 33). These data are confounded by the simultaneous presence of lower CD4 cell counts and viral phenotypic markers potentially associated with adverse clinical outcomes in the patients who rapidly develop drug-resistant HIV-1 isolates (2, 29, 33). Similar confounding variables will likely be seen in patients with isolates that show in vitro resistance to ddI and ddC. The clinical significance of decreasing drug susceptibility to ddI and ddC with ZDV-resistant clinical HIV-1 isolates remains to be determined. The ACTG 116B/117 trial comparing ZDV with ddI therapy in patients who had previously received at least 16 weeks of ZDV therapy showed a therapeutic benefit for asymptomatic patients and those with AIDS-related complex who switched to ddI but not those with AIDS (13). The benefit of ddI therapy did not increase with increasing time on ZDV therapy. These results may be consistent with parallel rises in ZDV, ddI, and ddC resistance with prolonged ZDV therapy. Patients with AIDS develop ZDV resistance more rapidly than patients with higher CD4 counts, and many AIDS patients may have entered the 116B/117 trial with HIV-1 isolates with decreased susceptibility to both drugs. Parallel rises in ZDV and ddI resistance may therefore lead to a fixed benefit of switching from ZDV to ddI despite an increasing percentage of patients with ZDV and cross-resistant isolates with longer periods of ZDV therapy. While our studies show a correlation between ZDV and ddI susceptibilities, the clinical significance of this relationship remains to be answered by prospective virologic studies.

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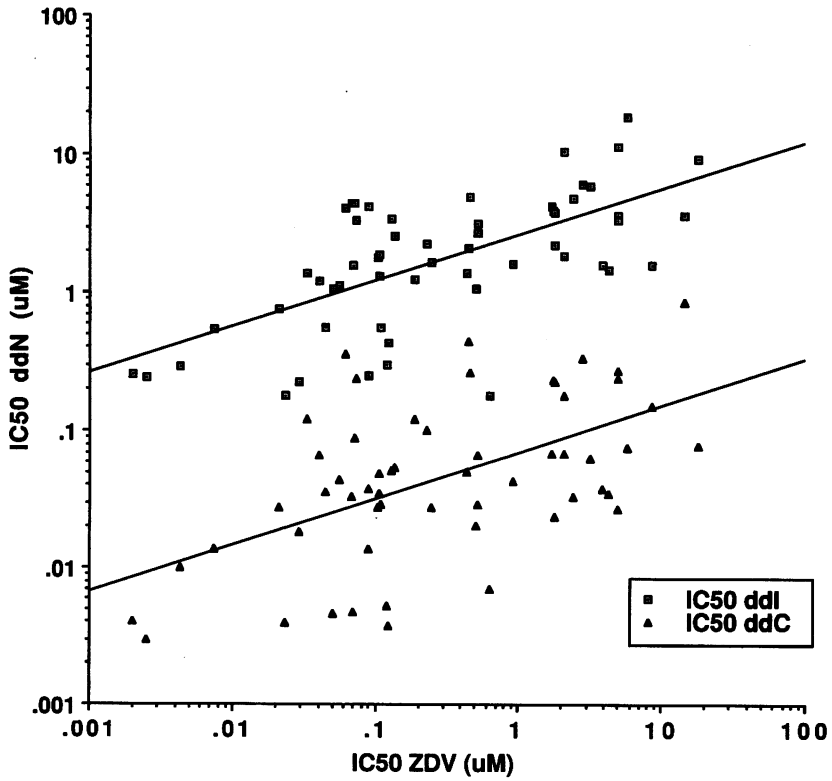

FIG. 2. Comparison of susceptibilities to ddI and ddC versus ZDV of HIV-1 isolates from MMCARR patients on ZDV monotherapy. All values represent IC_{50}s obtained by using the ACTG-DoD consensus drug susceptibility assay.

TABLE 5. Summary results for susceptibility to ZDV versus susceptibility to ddC by using primary HIV isolates in PBMC-based drug susceptibility assays

Group	*N*	*R*	Slope (SE)	Fold change	*P* value	95% Confidence interval of fold change
MMCARR	67	0.53	0.33 (0.11)	2.1	0.0001	1.3, 3.5
DM	15	0.46	0.12 (0.065)	1.3	0.085	1.0, 1.8
Total	82	0.52	0.29 (0.09)	2.0	0.0001	1.3, 3.0

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