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The Same Mutation That Encodes Low-Level Human Immunodeficiency Virus Type 1 Resistance to 2′,3′-Dideoxyinosine and 2′,3′-Dideoxycytidine Confers High-Level Resistance to the (−) Enantiomer of 2′,3′-Dideoxy-3′-Thiacytidine

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Variants of human immunodeficiency virus type 1 that display 500- to 1,000-fold resistance to the (−) enantiomer of 2′-deoxy-3′-thiacytidine and ≈4- to 8-fold resistance to 2′,3′-dideoxycytidine and 2′,3′-dideoxyinosine have been generated through in vitro selection with the former compound. The polymerase regions of several of these resistant viruses shared a codon alteration at site 184 (ATG → GTG; methionine → valine), a mutation previously associated with low-level resistance to 2′,3′-dideoxyinosine and 2′,3′-dideoxycytidine. The biological relevance of this mutation for the (−) enantiomer of 2′-deoxy-3′-thiacytidine was confirmed by site-directed mutagenesis with the HXB2-D clone of human immunodeficiency virus type 1.

Human immunodeficiency virus (HIV)-infected patients who receive prolonged therapy with antiretroviral drugs have been reported to develop strains of HIV that display resistance to these compounds (7, 13, 17). Resistant viruses can also be selected for by tissue culture passage, through gradual increases in the concentrations of drugs in the culture medium (4, 10).

The reverse transcriptase (RT) of HIV type 1 (HIV-1) displays considerable infidelity during the replication of viral RNA (12). Mutations at sites 41, 67, 70, 215, and 219 in the RT coding region are responsible for resistance to 3′-azido-3′-deoxythymidine (AZT) (6, 8), while mutations at sites 74 and 184 are associated with resistance to 2′,3′-dideoxyinosine (ddI) and cross-resistance to 2′,3′-dideoxycytidine (ddC) (5, 17).

We have now used in vitro passage and drug selection to generate HIV variants that are resistant to the (−) enantiomer of 2′-deoxy-3′-thiacytidine (3TC) and BCH-189, the racemic mixture of 3TC and the (+) enantiomer of 2′-deoxy-3′-thiacytidine, each of which possesses antiviral activity (1, 3, 15, 16). These drugs were provided by Glaxo Group Research, Greenford, United Kingdom, and Biochem Pharma, Montreal, Quebec, Canada, respectively. The 3TC preparation used was found crystalographically pure. AZT was a gift of Burroughs Wellcome, Inc., Montreal, Quebec, Canada. ddI and ddC were both purchased from Sigma Chemical Co., St. Louis, Mo.

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Viruses and cells. Both wild-type HIV-1 and resistant variants of HIV-1 were propagated on MT-4 cells as described previously (3, 4). Both clinical isolate 126, obtained from an HIV-1-infected subject prior to the initiation of antiviral chemotherapy, and the HIV-III_B laboratory strain of HIV-1 (kindly supplied by R. C. Gallo, National Institutes of Health, Bethesda, Md.) were used. We also studied the HXB2-D clone of full-length infectious DNA by using site-directed mutagenesis (2).

Selection process. MT-4 cells were incubated for 30 min with subinhibitory concentrations of drug and then were infected with either HIV-III_B or clinical isolate 126 of HIV-1 at a multiplicity of infection of 0.01. After 3 h of adsorption, the cells were washed and maintained in tissue culture medium, which contained the same subinhibitory dose of drug as that used during preincubation. The medium was changed twice weekly; each replacement contained a gradually increasing drug concentration, following the schedule shown in Table 1. Culture fluids (0.5 ml) from each round of viral replication were used to infect fresh MT-4 cells as described previously (3). Cells were monitored for the presence of RT activity and viral p24 antigen by an enzyme assay and an antigen capture assay (Abbott Laboratories, North Chicago, Ill.), respectively, as described previously (5). The ability of virus to replicate at a variety of drug concentrations was determined, and 50% effective concentrations (EC_{50}s) were calculated on the basis of RT activity and/or p24 antigen levels in culture fluids (5).

Significant levels of HIV-III_B resistance to 3TC and BCH-189 were apparent within 2 and 3 weeks, respectively, following initiation of the selection process on the schedule shown in Table 1. After replication in increasing concentrations of drug over a 9-week period, it was apparent that viral variants that were capable of growing in the presence of 500- to 1,000-fold the usual inhibitory concentrations of 3TC and BCH-189 could be selected for. EC_{50}s for viruses selected for during eight cycles of infection are shown in Table 2, which also contains information on the ability of the selected

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TABLE 1. In vitro selection for 3TC-resistant variants of HIV-1

Cycle of infection	Duration of infection cycle (days)	Concn (μM) at each cycle of:		EC_{50} (μM)[a] of the indicated drug for HIV-III_B selected for in:			
				BCH-189		3TC	
		BCH-189	3TC	3TC	BCH-189	3TC	BCH-189
1	7	1.5	3.0	0.9 ± 0.1	0.7 ± 0.08	1.2 ± 0.1	0.9 ± 0.07
2	7	3.0	6.0	1.6 ± 0.3	1.1 ± 0.2	4.6 ± 0.5	2.1 ± 0.3
3	7	5.0	10.0	2.8 ± 0.5	2.9 ± 0.2	7.0 ± 1.7	5.8 ± 0.8
4	7	10.0	20.0	ND	ND	ND	ND
5	8	50.0	100.0	25.5 ± 4.1	18.0 ± 1.4	61.8 ± 7.2	22.6 ± 2.9
6	8	200.0	200.0	ND	ND	ND	ND
7	9	400.0	400.0	268 ± 36	144 ± 15	385 ± 30	257 ± 19
8	9	800.0	800.0	910 ± 104	489 ± 35	936 ± 74	518 ± 56

[a] The results were calculated on the basis of p24 antigen levels in culture fluids. Data are means ± standard deviations for three replicate samples. ND, not determined.

viruses to replicate in the presence of ddI, ddC, and AZT. Some degree of cross-resistance was apparent for ddC, 3TC, ddI, and BCH-189. In contrast, none of the resistant isolates displayed significant resistance to AZT.

Cloning and sequencing. The complete RT coding regions of four 3TC-resistant variants of HIV-III_B and clinical isolate 126 were amplified with the RT01-RT02 primer pair, which was also used to determine the orientation of a mutated *Bal*I fragment inserted into wild-type HXB2-D (5). Primers used to discriminate wild-type from mutated sequences at codon Val-184 in the RT coding region included 184G, 184W, 184U, and 184D and have been described (5). Cloning and sequencing revealed the consistent presence of a mutation at position 184 (ATG → GTG; methionine → valine). This is the same mutation as that previously identified by our group as causing resistance to ddI (5). In addition, a second mutation, at position 89 (GAA → GGA; glutamine → glycine), was detected on three separate occasions. Coincidentally, this is the same mutation as that described as conferring resistance of the HIV-1 RT to ddGTP (11).

Site-directed mutagenesis. The biological significance of the site 184 mutation was confirmed by use of site-specific mutagenesis to substitute GTG for ATG at position 184 in recombinant clone HXB2-D as previously described (5). The virus generated was termed HXB2-D184 and was compared with HXB2-D for susceptibility to 3TC, BCH-189, ddI, and ddC. Table 2 shows that the GTG substitution at position 184 caused a diminution in susceptibility to 3TC, BCH-189, ddI, and ddC. EC_{50}s were calculated from levels of RT activity in culture fluids. Table 2 also contains data on AZT EC_{50}s calculated for HXB2-D and HXB2-D184. Studies on the potential biological significance of the site 89 mutation with regard to 3TC are in progress.

The current work shows that the site 184 mutation (methionine → valine) can account for drug resistance to BCH-189 and 3TC, which is currently being tested in early-phase clinical trials. It is relevant that amino acid 184 is situated in a highly conserved region of the viral RT, in which the sequence from positions 183 to 186 has been reported to be essential for proper enzymatic function. Mutations introduced at position 184 by site-directed mutagenesis have been reported to cause reductions in enzyme activity of over 80% for methionine → tyrosine and methionine → leucine substitutions (9).

The low-level cross-resistance demonstrated here for 3TC, ddI, and ddC may be due to the fact that each of these drugs possesses 2',3'-dideoxy moieties. The failure to demonstrate cross-resistance to AZT may be due to the 3'-azido moiety of this compound, although work with other azido derivatives and thymine derivatives will be necessary to confirm this suggestion. Other workers have described a valine mutation at position 74 that is associated with cross-resistance to ddI and ddC (17). Mutations at sites 103, 181, and 188 are associated with resistance to nonnucleoside inhibitors of the viral RT (10, 14).

The levels of resistance to 3TC reported here are as high as 1,000-fold above background levels. These levels are in contrast to the much lower levels of resistance to ddI and ddC attributable to the same site 184 mutation. Previous work showed that HXB2-D184 can be passaged onto cord blood lymphocytes and that the site 184 mutation can persist in such cells (6); thus, the site 184 mutation is not unique to MT-4 cells and can persist in primary cell types as well. Of course, it will also be important to determine whether this mutation can be selected for in primary cells.

It should be noted that the levels of resistance to BCH-189

TABLE 2. Susceptibility of drug-resistant variants of HIV-1 to antiviral compounds

Virus	EC_{50} (μM)[a] of:				
	3TC	BCH-189	ddC	ddI	AZT
Wild-type HIV-III_B	0.8 ± 0.1	1.5 ± 0.1	0.45 ± 0.3	19 ± 1.1	0.04 ± 0.006
3TC-selected HIV-III_B	936 ± 74	518 ± 56	2.5 ± 0.2	76 ± 9.2	0.05 ± 0.005
BCH-189-selected HIV-III_B	910 ± 104	485 ± 35	2.6 ± 0.2	70 ± 6.3	0.03 ± 0.002
Wild-type isolate 126	0.7 ± 0.06	1.8 ± 0.3	0.35 ± 0.04	22 ± 2.6	0.08 ± 0.01
3TC-selected isolate 126	1,050 ± 95	720 ± 41	2.8 ± 0.2	82 ± 6.4	0.04 ± 0.006
BCH-189-selected isolate 126	910 ± 80	680 ± 58	2.3 ± 0.3	65 ± 7.2	0.06 ± 0.004
HXB2-D	0.7 ± 0.08	1.7 ± 0.2	0.5 ± 0.03	17 ± 2.1	0.05 ± 0.003
HXB2-D184	1,080 ± 110	550 ± 36	2.2 ± 0.2	75 ± 6.0	0.06 ± 0.005

[a] The results were calculated on the basis of p24 antigen levels in culture fluids. Data are means ± standard deviations for three replicate samples.

and 3TC found in this study greatly exceeded those recently reported by our group (3). However, the previous work used a peak dose of 25 μM BCH-189 during the selection process, whereas the current study used concentrations as high as 800 μM. This difference in procedure probably accounts as well for the fact that the degree of cross-resistance to ddC was not as high as that previously observed (3). In addition, the current study shows that resistance to 3TC can apparently be selected for more rapidly in tissue culture than can that to BCH-189. This result may reflect some potential for antiviral synergy between the (+) enantiomer of 2′-deoxy-3′-thiacytidine and 3TC (1, 15).

Finally, it should be emphasized that the site 184 mutation (methionine → valine) was initially demonstrated on the basis of tissue culture selection with ddI and not 3TC (5). The current findings, showing a much higher level of resistance to 3TC, emphasize how work with a given drug may occasionally lead to results of greater significance for compounds other than that initially tested.

It will be of compelling interest to monitor patients treated with 3TC to determine whether HIV drug resistance emerges under conditions of clinical trials.

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ADDENDUM IN PROOF

Since acceptance of this manuscript a report has appeared in the literature (R. F. Schinazi, R. M. Lloyd, Jr., M.-H. Nguyen, D. L. Cannon, A. McMillan, N. Ilksoy, C. K. Chu, D. C. Liotta, H. Z. Bazmi, and J. W. Mellors, Antimicrob. Agents Chemother. **37**:875–881, 1993) describing the use of tissue culture selection to generate HIV-1 variants resistant to 3TC and BCH-189. This work also identifed the 184 RT mutation as present in both the genomes of such viruses and the peripheral blood mononuclear cells of a patient treated with 3TC. Our report confirms the biological relevance of this mutation for 3TC by site-directed mutagenesis.

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