ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1991, p. 988–991
0066-4804/91/050988-04$02.00/0

Vol. 35, No. 5

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

RONALD ROOKE, MICHAEL A. PARNIAK, MICHEL TREMBLAY, HUGO SOUDEYNS, XUGUANG LI, QING GAO, X.-J. YAO, AND MARK A. WAINBERG*

Lady Davis Institute—Jewish General Hospital and McGill AIDS Centre and Departments of Medicine and Microbiology, McGill University, Montreal, Quebec, Canada H3T 1E2*

Received 10 September 1990/Accepted 14 February 1991

We used a viral endpoint dilution assay to show changes in the proportion of zidovudine (azidothymidine; AZT)-resistant viruses within a heterogeneous mixture of human immunodeficiency virus type 1 (HIV-1) quasispecies isolated from patients on long-term AZT therapy. Several HIV-1 isolates, which could replicate in 10 μM AZT, were susceptible to both 2′,3′-dideoxycytidine and a novel cytosine analog BCH-189, in which a sulfur atom replaces the 3′ carbon of the pentose ring. In certain instances, cross-resistance was seen with 3′-didehydro-2′,3′-dideoxythymidine. Although most strains of AZT-resistant HIV-1 displayed reduced susceptibility to 3′-azido-2′,3′-dideoxyuridine, two strains were identified for which this was not the case.

Both we and other investigators have described the isolation of zidovudine (azidothymidine; AZT)-resistant variants of human immunodeficiency virus type 1 (HIV-1) from patients on long-term drug therapy (4, 6, 10), both by primary isolation of virus in the presence of drug and by showing that frozen samples of HIV-1, first isolated in the absence of drug pressure, were able to replicate efficiently when AZT was added to the tissue culture medium. The outgrowth of such viruses could have clinical relevance, since AZT has proven efficacy in the retardation of HIV-1-associated disease (2). It is important to determine the frequencies with which variants of HIV-1 resistant to AZT and other drugs occur; it is also important to determine their susceptibilities and cross-resistances to other nucleoside analogs.

Blood samples were obtained at random from HIV-infected individuals both prior to and at various intervals following initiation of AZT therapy. For each blood sample obtained, peripheral blood mononuclear cells (PBMCs) (5×10^6 cells) were isolated by Ficoll-Isopaque gradient centrifugation and cocultivated with cord blood lymphocytes (CBLs; 5×10^6 cells) in RPMI 1640 medium supplemented with 10% fetal bovine serum and in phytohemagglutinin type P-1 (0.1%) (8, 10). After 3 days, the cells were washed, resuspended to a concentration of 10^6/ml in growth medium containing interleukin 2 (5% [vol/vol]), and maintained by routine splitting of the cultures at 2- to 3-day intervals. Viral replication was regularly monitored in culture fluids by assaying for the presence of reverse transcriptase (RT) activity and HIV-1 p24 antigen (11). This procedure routinely yields isolation of HIV-1 in over 85% of cases in our laboratory from patients categorized in Centers for Disease Control categories II to IV. Culture fluids showing the presence of HIV-1 were clarified and frozen at −70°C.

Primary screening of blood samples for HIV drug resistance. Portions of CBLs (5×10^6 cells per ml) were pretreated with 7.5 μM AZT for 4 h prior to inoculation in the presence of 7.5 μM AZT with 0.5 ml of RT-positive culture fluid harvested from cocultures of PBMCs and CBLs, as described above. Parallel cultures of CBLs did not contain drug. Fresh medium, including AZT when appropriate, was added three times weekly. Phytohemagglutinin-stimulated CBLs (5×10^5 cells) were added to the cultures every 7 days. RT activity was determined on clarified culture fluids every 3 to 4 days. Of 46 blood samples from 72 subjects on AZT therapy for at least 6 months, 34 (74%) were found to contain viruses that were able to replicate in the presence of drug (data not shown). This is a far higher percentage of such blood samples than that which we reported previously (10).

To determine whether any of the frozen isolates obtained in the absence of AZT had a reduced affinity for AZT, four consecutive frozen culture fluids from each patient screened were thawed and used to infect the PBMCs of a normal donor. The same amount of RT activity (about 50,000 cpm, corresponding to about 100 tissue culture infectious doses [$TCID_{50}$s] of infectious virus in each case) taken from the clarified culture fluids of the secondary PBMC passage of each isolate were serially diluted 1:2, in duplicate, into microwells containing PBMCs from the same healthy donor (2.5×10^6 cells per well). The PBMCs of one series of wells were preincubated for 4 h prior to viral addition in medium containing 7.5 μM AZT; these cells were maintained in 7.5 μM both during and following viral inoculation. The other series were grown in the absence of drug throughout the experiment. Other controls were uninfected cells grown in the presence or absence of AZT. After 10 days, cultures were examined for the presence of viral p24 antigen by indirect immunofluorescence assay (IFA) (10).

Figure 1 shows the capacity of sequential viral isolates obtained from four different subjects, selected at random, to grow in PBMCs in 7.5 μM AZT. While all isolates were capable of replication in the absence of AZT, only one (patient B) was capable of establishing infection at a high dilution in the presence of drug. Virus which was isolated

* Corresponding author.

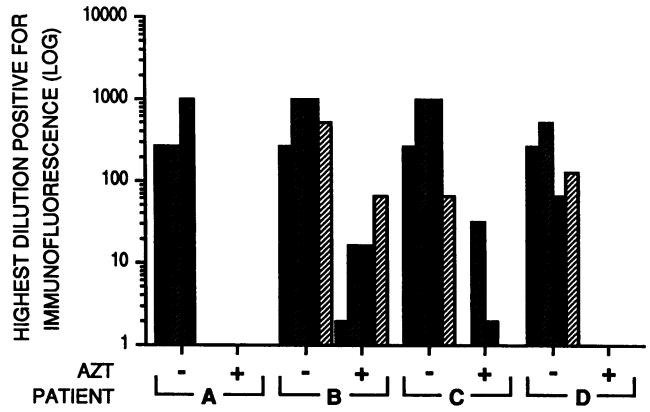

FIG. 1. Isolation of AZT-resistant variants of HIV-1 from sequential cocultures of cells from the same patient. Sequential isolates from four patients (patients A to D) were screened for the occurrence of AZT-resistant viruses for each of weeks 0 (■), 32 (▨), 46 (▩), and 52 (▨) of the patient's AZT treatment (with the exception of patient A, for whom the isolate of week 52 was unavailable). The highest dilutions at which each virus could establish infection in the presence and absence of AZT were determined by IFA for the presence of viral p24 antigen. Cultures were considered positive when greater than 5% of cells were IFA positive for p24 antigen.

from this same patient prior to initiation of AZT treatment (week 0, isolate B-0) was able to replicate in the presence of drug only at a very low dilution (1:2). HIV-1, which was isolated from the same subject after 52 weeks of treatment (B-52), could be diluted to 1:64 and still grow in 7.5 μM AZT. Thus, this technique reveals changes in the proportion of resistant virus within the heterogeneous mixture of HIV-1 quasispecies isolated from this patient over time. Similar findings were obtained by examining these various culture fluids for the presence of viral RT activity and p24 antigen.

The technique described here of using serial dilutions of virus to assess the proportion of AZT-resistant species may prove useful in monitoring individual patients longitudinally and in correlating clinical status with the development of drug resistance. All four patients thus monitored were relatively asymptomatic prior to commencement of therapy (Centers for Disease Control class III) and have not progressed to advanced HIV-1-associated disease or AIDS.

The AZT-resistant variants may have represented only a small fraction of the HIV-1 species present in the culture fluids tested. In the absence of AZT, all strains grew, while the presence of drug inhibited the replication of AZT-susceptible viruses only. This could lower the effective multiplicity of infection by a factor proportional to the fraction of AZT-resistant viruses in the pool. A decrease in the multiplicity of infection could result in an increased time to culture positivity. Several groups have reported that AZT-resistant strains of HIV-1 can be isolated without including drug during primary isolation procedures (4, 5, 10). Although the presence of drug did not affect cell viability in our experiments, we cannot rule out the possibility that the longer times needed for culture positivity and the lower titers of RT activity observed in the case of AZT-treated cultures were due to the effects of AZT on cellular metabolism. The time dependence of the emergence of viruses possessing increasing levels of AZT resistance in patient B (Fig. 1) suggests that multiple viral mutations might have occurred in this individual, consistent with recent observations on the isolation of AZT-resistant viruses from patients with AIDS on long-term AZT therapy (5, 7, 10). It is possible that we may have amplified the presence of AZT-resistant virus from the preexisting viral pool.

Susceptibility to other nucleosides of AZT-resistant variants of HIV-1. MT-4 cells growing in the logarithmic phase (10^6 cells in 1 ml of supplemented RPMI 1640 medium) were pretreated for 4 h with a variety of concentrations of AZT (0 to 20 μM) or one of four other nucleoside analogs: 2',3'-dideoxycytidine (ddC; Sigma Chemical Corp., St. Louis, Mo.), 2',3'-dideoxy-3'-didehydrothymidine (d4T; Bristol-Myers Inc., Wallingford, Conn.), BCH-189 (2'-deoxy-3'-thiacytidine; in which the 3' carbon of the pentose ring is replaced by an S atom; IAF Biochemicals, Montreal, Quebec, Canada), and 3'-azido-2',3'-dideoxyuridine (AZDU; Triton BioSciences, San Francisco, Calif.). Thereafter, 50 μl of cell suspension was plated into quadruplicate wells of a 96-well plate containing growth medium, including the same concentration of compound used for pretreatment, and drug-resistant HIV-1 (100 $TCID_{50}$s; final volume, 200 μl). After 7 days of incubation, the cells were examined for p24 antigen by IFA. For determination of the $TCID_{50}$, 25 μl of the viral suspensions whose titers were to be determined was subjected to 10-fold serial dilutions in RPMI 1640 medium into 96-well microtiter plates. To each well was added 75 μl of RPMI 1640 medium containing 5×10^4 MT-4 cells in the logarithmic phase of growth. After 4 days of incubation at

TABLE 1. Susceptibilities of various HIV-1 isolates to different nucleosides

Virus tested	ID_{50} (μM)[a]				
	AZT	ddC	BCH-189	d4T	AZDU
$HIV\text{-}111_B$	0.05 ± 0.006	0.45 ± 0.05	0.25 ± 0.028	1.6 ± 0.14	0.6 ± 0.07
B-0	0.05 ± 0.003	0.50 ± 0.03	0.4 ± 0.03	1.5 ± 0.18	NT[b]
B-52	0.50 ± 0.04	0.6 ± 0.09	0.55 ± 0.04	12.4 ± 1.6	NT
AZT-resistant strain A-161	1.2 ± 0.10	0.5 ± 0.04	0.3 ± 0.02	1.4 ± 0.18	8.6 ± 1.1
AZT-resistant strain C-246	0.7 ± 0.09	0.4 ± 0.04	0.45 ± 0.04	1.1 ± 0.09	10.3 ± 1.0
AZT-resistant strain G-68	0.65 ± 0.05	0.3 ± 0.05	0.3 ± 0.06	1.2 ± 0.09	0.5 ± 0.06
AZT-resistant strain R-102	1.2 ± 0.11	0.5 ± 0.04	0.55 ± 0.07	1.0 ± 0.09	0.3 ± 0.05
AZT-resistant strain X-78	1.1 ± 0.12	0.4 ± 0.06	0.35 ± 0.05	8.8 ± 0.95	0.5 ± 0.03
AZT-susceptible strain S-26	0.02 ± 0.003	0.6 ± 0.04	0.4 ± 0.03	1.2 ± 0.09	0.6 ± 0.04

[a] ID_{50} calculations were based on production of viral RT activity in HIV-infected MT-4 cultures. Values are means ± standard deviations.
[b] NT, Not tested.

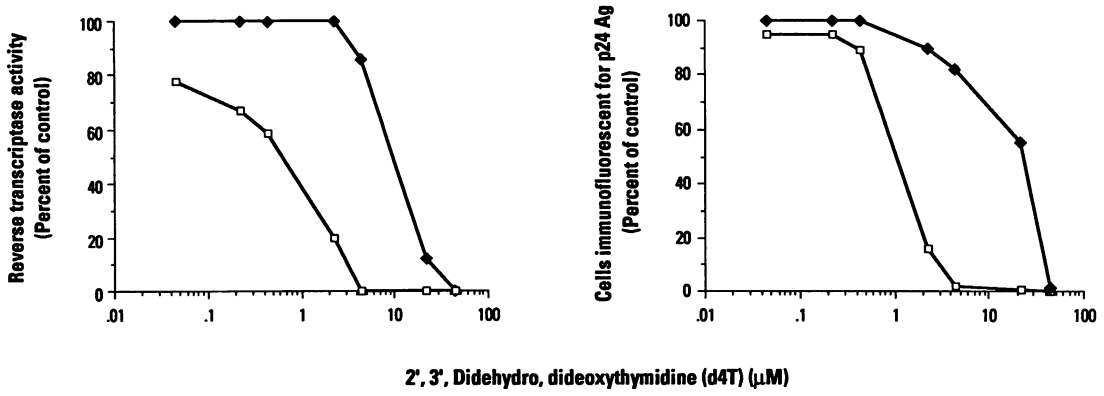

FIG. 2. Susceptibility to d4T of each of the B-0 (◆) and B-52 (◇) strains of HIV-1 grown in MT-4 cells. Cells infected in the absence of drug served as controls. Data for each drug concentration are expressed as the percentage of RT activity or p24 antigen (Ag)-positive cells found in control cultures.

37°C, the cells were harvested, and infection was determined by IFA of the p24 antigen.

Neither HIV-III_B nor the B-0 isolate was able to replicate under conditions of 0.1 μM AZT (data not shown). The concentration of AZT that inhibited both RT activity and p24 antigen expression by 50% (i.e., ID_{50}) was 0.05 μM for each of these isolates (Table 1). In contrast, the B-52 isolate was able to infect MT-4 cells, even when AZT was present at 10 μM. The ID_{50} for the B-52 isolate was calculated to be 0.5 μM AZT. Most analogs were able to inhibit replication of both the AZT-resistant B-52 strain and a number of other isolates with diminished susceptibilities to AZT. However, the B-52 strain and 1 of 11 isolates tested displayed marked resistance to d4T (Table 1 and Fig. 2). In addition, two of these isolates were susceptible to AZDU. These observations differ from those reported previously by other workers who studied AZT-resistant variants (5, 6). In this regard, it is noteworthy that d4T, AZT, and AZDU are structurally similar. We are performing cross-resistance experiments on a greater number of AZT-resistant isolates to substantiate these findings, which underline the heterogeneous nature of clinically derived isolates and which may be due to undescribed mutations within the HIV-1 genome. All isolates were susceptible to ddC and BCH-189.

We determined K_i and ID_{50}s of AZT-triphosphate (AZT-TP) for each of four different AZT-resistant strains, including those showing cross-resistance to d4T and their AZT-susceptible parental counterparts obtained from the same donors prior to therapy (6). No significant differences in either K_i or ID_{50} for AZT-TP were noted between these AZT-susceptible and -resistant pairs. These data are consistent with results reported by other workers (5, 7) and presumably reflect the fact that the microenvironment of the cell and/or the virion, in which AZT-TP inhibits RT activity intracellularly, may differ significantly from test tube conditions, in which no significant effect can be demonstrated.

We need to acknowledge that the characterizations reported here were not performed with plaque-purified AZT-resistant or -susceptible strains. In fact, it is unlikely that different results would have emerged had this been done. The error rate of viral RT is about 1 misincorporation per 10^4 bases; hence, amplification of plaque-purified virus to quantities necessary for analysis would involve extensive replication which would have resulted in heterogeneity in the *pol* gene as well as other areas of the viral genome (1, 3, 9, 12).

(Most aspects of this research were performed by Ronald Rooke in partial fulfillment of the requirements of the Ph.D. degree, Faculty of Graduate Studies, McGill University.)

This work was supported by grants to M.A.W. and M.A.P. from Health and Welfare Canada.

We thank Rhona Rosenzweig for typing the manuscript and Christine Lalonde for preparing the figures.

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