HIV with Reduced Sensitivity to Zidovudine (AZT) Isolated During Prolonged Therapy

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The drug sensitivities of human immunodeficiency virus (HIV) isolates from a group of patients with acquired immunodeficiency syndrome (AIDS) or AIDS-related complex (ARC) who were receiving zidovudine (3'-azido-3'-deoxythymidine, AZT) therapy were tested by means of a newly developed plaque assay in CD4+ HeLa cells. Fifty percent inhibitory dose (ID50) values of 18 isolates from untreated individuals ranged between 0.01 μM and 0.05 μM. In contrast, most isolates from patients who had received zidovudine for 6 months or more exhibited decreased sensitivity characterized by changes in ID50 or ID95 values (or both), with isolates from several patients (5/15) showing 100-fold increases in ID50. The latter isolates were also insensitive to 3'-azido-2',3'-dideoxyuridine; however, the isolates were still sensitive to 2',3'-dideoxyxytidine, 2',3'-dideoxy-2',3'-didehydrothymidine, or phosphonoformate. It cannot be determined from this small sample of patients whether development of a less sensitive virus phenotype results in clinical resistance. Appearance of such variants was not associated with a consistent increase in viral p24 concentrations in patient plasma and did not herald any sudden deterioration in clinical status. More extensive studies are required to determine the clinical significance. Thus, it would be premature to alter any treatment protocols for HIV-infected individuals at present.

Zidovudine is effective against HIV in vitro and has been demonstrated to improve the quality and length of life of patients with AIDS and advanced ARC (1, 2). Furthermore, serum levels of viral p24 antigen are reduced after initiation of drug treatment, suggesting a significant antiviral effect (3, 4). However, dose reductions may be required because of an inability of patients to tolerate the drug (5, 6). In addition, virus can be isolated during therapy even with current dose regimens (1, 4), which indicates that zidovudine may not suppress virus production completely in vivo. The objective of the present study was to investigate whether prolonged exposure of HIV to zidovudine in patients might result in selection of variants with reduced drug sensitivity. Attempts to select such variants by passage in tissue culture have so far been unsuccessful (7).

HIV isolation was attempted from 101 patients; 54 of these were receiving zidovudine therapy as a result of enrollment between March 1986 and July 1987 in three separate, controlled trials at the University of California, San Diego. Isolates were made by co-cultivation of peripheral blood lymphocytes (PBLs) (8), from patients before and during prolonged therapy, with cells of the continuous cell line MT-2 (9). Where isolation was unsuccessful, attempts were made to recover virus from PBLs stored before initiation of therapy. Forty-six high-titer virus stocks were produced from a total of 33 individuals, 21 of whom had received zidovudine for up to 30 months. Virus sensitivity data were obtained from 17 of those patients (2 of whom were treated for less than 6 months). Of the 46 isolates, 18 were from patients who had received no treatment at the time of isolation. The success rate for virus isolation from PBL samples, whether fresh or frozen, from treated or untreated individuals, was approximately 30% in each case.

A HeLa cell line (HT4-6C) expressing the human CD4 receptor on its surface (9) was used to establish an assay system for assessing the drug sensitivity of clinical isolates of HIV (11). The majority of the 46 isolates obtained in MT-2 cells (44/46) readily formed syncytial foci of infection (plaques) in HT4-6C cells, thus permitting measurement of drug sensitivity by plaque reduction assay. Isolates from 18 individuals who had not been treated with zidovudine (comprising patients with AIDS or ARC, and a small number of asymptomatic seropositive individuals) were shown to be remarkably similar in sensitivity; ID50 values for zidovudine inhibition were in the range of 0.01 to 0.05 μM with a mean value of 0.03 μM (Table 1). These data provided the baseline for investigation of isolates from patients treated with zidovudine.

Isolates from patients treated for less than 6 months were indistinguishable in sensitivity from isolates from untreated patients. Most isolates obtained after longer periods of therapy showed decreases in sensitivity to zidovudine (Table 1 and Fig. 1). The changes were, in some cases, quite small with ID50 values in the range observed for pre-therapy isolates but with significantly higher ID95 values. This tail on the inhibi-
fact of the modified HeLa cells, we measured the sensitivity by means of an assay based on inhibition of cytopathic effects in MT-2 cells (12). In these experiments, we used paired isolates from five individuals in whom the greatest increases in ID_{50} values had been observed in the HeLa system. The relative changes in sensitivity were similar (Table 2) and these were confirmed by measurement of p24 levels in culture supernatants from MT-2 cells. However, the absolute values for ID_{50} obtained in MT-2 were different from those in HeLa cells, a common observation when comparing different assay systems in which different cells, end points, and input multiplicities are used.

By means of the five paired isolates we asked whether the development of in vitro resistance to zidovudine extended to other anti-retroviral agents. Post-therapy isolates that were resistant to zidovudine were also resistant to 3'-azido-2',3'-dideoxycytidine (AZdU), a closely related compound; in contrast, the same isolates displayed sensitivities to 2',3'-dideoxy-xycytidine (ddC), 2',3'-dideoxy-2',3'-dideoxythymidine (D4T), and phosphonoformate (PFA) that were similar to those of isolates obtained near the initiation of therapy, with the possible exception of the sensitivity of P022C to D4T (Table 2).

The most likely mechanism for decreased sensitivity to zidovudine would appear, on the basis of current knowledge, to be through mutation in the reverse transcriptase (RT) gene (13). We therefore tested virion-associated RT from the five paired isolates for inhibition by zidovudine triphosphate (14). These experiments revealed no apparent difference in the degree of inhibition, as the measured ID_{50} values all fell in the narrow range from 0.005 μM to 0.009 μM. These data suggested that no changes in affinity of the enzymes for zidovudine triphosphate had occurred. It remains possible that mutations in RT could result in a decreased rate of incorporation of the analog into DNA. More substantial amounts of RT from these isolates are currently being obtained, by expression of cloned RT genes in Escherichia coli, to facilitate detailed biochemical characterization of these enzymes.

Of the 17 patients who received therapy, six discontinued treatment in the first 6 months (five died within 18 months). Clinical data for the remaining 11 patients (treated for 15 months or more) are shown in Fig. 3. Five of these yielded viruses with marked reductions in sensitivity (increase in ID_{50} values of 100-fold or more; two of the patients had received full-dose therapy throughout, and the remainder had reduced or interrupted dosing (Fig. 3A). Less dramatic changes were seen in isolates from the remaining six patients (Fig. 3B), all of whom received reduced or interrupted dosing schedules. At this stage we have insufficient data to know whether any particular pattern of therapy favors selection of less sensitive strains. Similarly, we do not know whether the amount of active virus replication is an important factor.

Experience with acyclovir, an established and successful treatment for herpes simplex virus (HSV) infections, has suggested that although resistance is rare it is more likely to develop in immunocompromised patients (16). In this study most patients were profoundly immunocompromised, with low CD4 cell counts throughout their therapy.
(mean values below 100 cells per milliliter). It will be necessary to study isolates from asymptomatic, seropositive individuals who have experienced prolonged zidovudine therapy to evaluate the effect of immune status on development of resistance.

The critical issue is whether the development of a less sensitive virus phenotype results in clinical resistance to drug therapy. Although the mean peak plasma level of zidovudine achieved 1.5 hours after a 250-mg oral dose is approximately 2.3 μM (16), the interpretation of the relation between plasma levels and the values determined in sensitivity tests remains to be established. HIV infection is characterized by chronic, persistent, virus replication, and disease is chronic, progressive, and extremely variable among patients. Consequently, it will be difficult to correlate emergence of resistant variants with changes in clinical status or other markers. For example, in patients with detectable serum p24 antigen at initiation of therapy (Fig. 3) there was an apparent early decrease in antigen level consistent with previous reports (1, 3), which had led to the belief that p24 antigen levels are correlated with virus replication. However, those patients who developed variants with marked reductions in sensitivity (for example, patients A012 and A018) had no consistent pattern of resurgent p24 antigen.

Furthermore, it has been shown that a significant fraction of patients who lack p24 antigen in their serum have circulating virus or viral antigen (or both) in immune complexes (17). The use of p24 antigen levels as a marker of virus replication in patients may

Fig. 3. Therapy and disease patterns of patients treated for more than 6 months. The oral dose of zidovudine indicated by the horizontal bar was either 250 mg (broad bar), 200 mg (intermediate), or 100 mg (narrow bar) given every 4 hours. The viruses isolated are classified either S (ID50 < 0.05 μM, ID95 < 1 μM), R (ID50 > 1 μM), or PR (all other isolates). ID50 values in brackets. Figures below the bars indicate serum p24 levels (pg/ml). Patient's death is indicated (D). (A) Three ARC patients (A012, A018, and A036) and two AIDS patients (P022 and P026) from whom HIV isolates of considerably reduced sensitivity were recovered after 9 to 26 months. At initiation of therapy, clinical parameters were as follows: CD4⁺ lymphocytes, median of 17 cells/ml (range 0–200 cells/ml); Karnofsky score, median of 100 (range 80–100); and body weight median of 68.3 kg (range 62.3–78 kg). There was little change during therapy. After 18 months' treatment the corresponding values were as follows: CD4⁺ lymphocytes, median of 16 cells/ml (range 0–164 cells/ml); Karnofsky score, median of 90 (range 70–100); and body weight, 67.9 kg (range 63.0–69.1 kg). (B) Six patients from whom HIV of reduced sensitivity was recovered over 12 to 27 months, including four ARC patients (A001, A025, A035, and A040), one AIDS patient (P043), and one patient with Kaposi's sarcoma (K017) are shown. Pretreatment clinical values were as follows: CD4⁺ lymphocytes, median of 72 cells/ml (range 7–600 cells/ml); Karnofsky score, median of 90 (range 80–100); and body weight, 68.5 kg (range 55.8–79.1 kg). After 18 months the corresponding values were as follows: CD4⁺, median of 45 cells/ml (range 22–458 cells/ml); Karnofsky score, median of 80 (range 80–90); and body weight, 65.3 kg (range 54–81 kg).
Since and pathogenic potential of these variants, since in general all HSV variants resistant to acyclovir that have been characterized exhibit attenuation of virulence (18). It remains to be determined whether a similar picture will emerge with HIV. Because of the gradual and progressive changes in the sensitivity of isolates from individual patients, alterations in clinical status with the emergence of resistant variants would be difficult to recognize. However, it is clear that appearance of such variants does not herald any sudden or rapid deterioration in clinical condition (Fig. 3).

In summary, HIV isolates from a group of 15 patients who had received zidovudine therapy for the treatment of AIDS or ARC for periods of at least 6 months showed some reduction in sensitivity to zidovudine in vitro when compared to isolates from patients who had not received the drug. Virus was isolated from 30% of patients, and this may not be truly representative. However, since changes in sensitivity have only been observed in viruses isolated from patients on zidovudine therapy, and not in viruses from patients at similar stages of disease progression who have received no drug, it is reasonable to assume that the less sensitive variants have been selected by exposure of HIV to zidovudine in the patient rather than by any peculiar selection pressures in transformed lymphocytes used for virus isolation. This conclusion is further supported by the failure of isolates with reduced zidovudine sensitivity to show cross-resistance to several other anti-retroviral agents.

In the absence of a clear picture of the clinical implications of these observations, which will require additional laboratory and clinical studies, it would be premature to alter any of the treatment protocols for HIV-infected individuals. However, HIV isolates from additional patients involved in clinical trials, including those involving other anti-retroviral agents, should be closely monitored, and it will be important to examine the potential utility of combined therapies.

REFERENCES AND NOTES

8. PBL samples were prepared by separation on "Ficoll-Hypaque" gradients and co-cultivated directly with MT-2 cells (approximately 10 of each) after pre-stimulation for 24 to 72 hours with phytohemagglutinin (3 μg/ml). In many cases PBLa were cultured after long-term frozen storage. Cultures were maintained in RPMI 1640, supplemented with 10% fetal bovine serum, antibiotics, 2% interleukin-2, and polybrene (2 μg/ml) and expanded by addition of fresh MT-2 cells when a cytopathic effect was observed (between 4 and 14 days). HIV replication was confirmed by the detection of p24 antigen in culture supernatants (p24 antigen detection kit, Abbott, Chicago, Illinois). Virus pools were prepared from infected cultures and stored in aliquots at −70°C. All drug sensitivities were determined with virus pools that had been passaged no more than twice from original cultures.
11. Inhibition of plaque formation (foci of multinucleated giant cells) was determined by infecting monolayers of HeLa HTA-6C cells (10) with cell-free HIV preparations. The input inoculum was adjusted to give 100 to 300 plaques per well (in 24-well plates) in the no-drug control cultures. Virus was allowed to adsorb for 1 hour at 37°C prior to the addition of inhibitor in the culture medium (Dulbecco’s modification of Eagle’s medium, containing 5% fetal bovine serum plus antibiotics). After 3 days of incubation, monolayers were fixed with 10% formaldehyde and stained with 0.25% crystal violet to visualize plaques. This staining procedure revealed obvious individual dense foci of multinucleated giant cells. ID50 values were derived directly from plots of percent plaque reduction versus inhibitor concentration.
14. Each virus was propagated in MT-2 cells (9) and clarified infected cell supernatants were prepared from cultures at peak p24 antigen levels (in excess of 10 pg/ml). Virus was precipitated from these supernatants at 4°C with polyethylene glycol (10%) and NaCl (0.1M). Pellets were washed with cold phosphate-buffered saline and virus was solubilized in buffer containing 0.5% triton X-100, 500 mM KC1, 50 mM tris-HCl pH 7.5, 1 mM phenylmethylsulfonyl fluoride, and 5 mM β-mercaptoethanol. RT activity was assayed with poly(A)·oligo(dT) primer template and [3H]dCTP (5 μM and 10 μCi/ml) as described by B. A. Lader, D. J. M. Purifoy, K. L. Powell, G. Darby, EMBO J. 6, 3133 (1987) and inhibition experiments using zidovudine triphosphate were performed as described (13).
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