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′-deoythymidine, AZT) therapy were tested by means of a newly developed plaque assay in CD4$^+$ HeLa cells. Fifty percent inhibitory dose (ID_{50}) values of 18 isolates from untreated individuals ranged between 0.01 μ*M* and 0.05 μ*M*. In contrast, most isolates from patients who had received zidovudine for 6 months or more exhibited decreased sensitivity characterized by changes in ID_{50} or ID_{95} values (or both), with isolates from several patients (5/15) showing 100-fold increases in ID_{50}. The latter isolates were also insensitive to 3′-azido-2′,3′-dideoxyuridine; however, the isolates were still sensitive to 2′,3′-dideoxycytidine, 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 successful, 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; ID_{50} values for zidovudine inhibition were in the range of 0.01 to 0.05 μ*M* with a mean value of 0.03 μ*M* (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 ID_{50} values in the range observed for pre-therapy isolates but with significantly higher ID_{95} values. This tail on the inhibi-

Table 1. Correlation of sensitivity of HIV isolates to zidovudine with duration of therapy. HIV isolates were obtained by direct cocultivation of patients' PBLs with MT-2 cells (*8*). Virus was titrated by plaque assay with HT4-6C cell monolayers and then assessed for zidovudine sensitivity by plaque reduction assay (*11*). ID_{50} values were determined directly from plots of percent plaque reduction versus zidovudine concentrations (log_{10}). All of the HIV isolates obtained during therapy were from patients with ARC or AIDS as were the majority of the 18 isolates from untreated individuals (7 with AIDS, 8 with ARC, and 3 asymptomatic).

Duration of therapy (months)	Number of isolates	ID_{50}		
		Mean (μ*M*)	Median (μ*M*)	Range (μ*M*)
None	18	0.03	0.03	0.01–0.05
1–5	6	0.03	0.03	0.007–0.05
6–11	8	1	0.6	0.06–4
12–17	8	1	0.07	0.04–6
18+	3*	3	2	0.1–6

*Two isolates obtained 2 months apart from one of the patients in the 18+ group showed the same sensitivity (ID_{50} values of 0.1 μ*M*). Only one value was included.

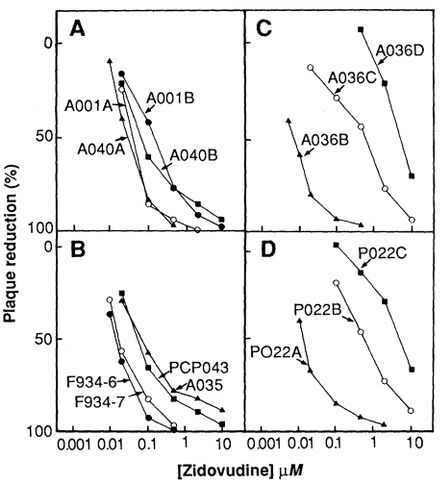

Fig. 1. Plaque reduction assays in HeLa HT4-6C cells. HIV isolates were obtained from untreated individuals or from patients receiving zidovudine (AZT) therapy for different periods of time (*8*), and zidovudine sensitivity was determined by plaque reduction assays (*11*). (**A**) The sensitivity of paired isolates from two individuals. Isolate A001A was obtained before treatment initiation and A001B after 12 months of therapy. A040A was a pretreatment isolate and A040B was obtained after 14 months of therapy. (**B**) The sensitivity of four unrelated isolates. F934-6 and F934-7 were obtained from untreated patients with AIDS and ARC, respectively. Isolate A035 was obtained after 14 months of therapy and PCP043 after 13 months of therapy. (**C**) and (**D**) show sensitivities of sequential isolates from two individual patients. Isolates A036B, A036C, and A036D were obtained after 2, 11, and 20 months of therapy (respectively) and isolates P022A, P022B, and P022C were obtained after 1, 11, and 16 months of therapy (respectively).

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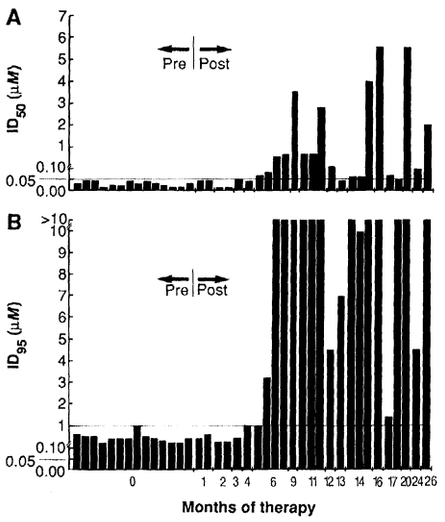

Fig. 2. Comparison of zidovudine sensitivities of HIV isolates obtained before and after initiation of treatment. HIV isolates were obtained from PBL samples as described in Table 1. Virus was propagated in MT-2 cells (*8*) and zidovudine sensitivity determined by plaque reduction assay with HeLa HT4-6C cell monolayers (*11*). The upper and lower parts of the figure depict matched ID_{50} and ID_{95} values for each isolate. The arrows indicate those isolates obtained before (Pre) or after (Post) initiation of zidovudine therapy, and the maximum ID_{50} and ID_{95} values observed for isolates from untreated individuals (0.05 μ*M* and 1 μ*M*, respectively) are indicated.

tion curve (Fig. 1), with or without a shift in ID_{50}, suggests the presence of a subfraction of insensitive variants in an otherwise sensitive population. Sensitivity profiles for paired isolates obtained before and during therapy from two of the individuals who displayed small changes in sensitivity are shown in Fig. 1A. In a similar example (Fig. 1B), isolates from two patients who had received therapy for 13 to 14 months had higher ID_{95} values than two unrelated patients who had not received therapy.

In other cases the changes in ID_{50} values were considerably higher with one-third (5/15) of the patients showing more than 100-fold increases in ID_{50} after 6 months or more of therapy. More than two sequential isolates were made in four patients and in each case there appeared to be a progressive decrease in drug sensitivity over an extended period of time (Fig. 1, C and D, show two examples). The sensitivity data for most isolates (including all posttreatment isolates), correlating ID_{50} and ID_{95} values with duration of therapy, are collated in Fig. 2. Many of the ID_{95} values were >10 μ*M*, the upper limit for this assay system, since above this drug concentration cytotoxic effects preclude accurate determination of plaque numbers.

To confirm that these shifts of in vitro sensitivity were not attributable to an artifact 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′-dideoxyuridine (AZdU), a closely related compound; in contrast, the same isolates displayed sensitivities to 2′,3′-dideoxycytidine(ddC), 2′,3′-dideoxy-2′,3′-didehydrothymidine (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 μ*M* to 0.009 μ*M*. 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

Table 2. Sensitivities of paired HIV isolates to antiviral agents. Isolates from different individuals showing the most significant decreases in sensitivity to zidovudine (>100-fold increase in ID_{50} value) were tested for sensitivity to other anti-retroviral compounds by plaque-reduction assay in HeLa HT4-6C cells. Matched isolates sensitive to zidovudine (obtained close to initiation of therapy) were assessed in parallel. The duration of therapy indicates the period each patient received zidovudine when PBL samples were taken. Also shown is the zidovudine sensitivity of isolate pairs assessed by inhibition of cytopathic effect in MT-2 cells (*12*). Absolute ID_{50} values for each isolate obtained after the start of therapy were not determined because of the toxic effects in MT-2 cells above 30 μ*M* zidovudine. Assessment of zidovudine sensitivity in MT-2 cells by measuring p24 antigen in the culture supernatant resulted in ID_{50} values of ≤0.03 μ*M* for all isolates obtained close to the initiation of therapy and ≥10 μ*M* for all isolates obtained during therapy. ND, not determined.

Isolate	Duration of therapy (months)	ID_{50} in HT4-6C (μ*M*)					ID_{50} in MT-2 (μ*M*)
		Zidovudine	AZdU	ddC	D4T	PFA	Zidovudine
A012B	2	0.01	0.4	0.06	2	14	0.3
A012D	26	2	89	0.2	2.5	28	>30
A018A	0	0.01	1.0	0.3	2	20	0.3
A018C	14	4	50	0.2	1.6	13	>30
A036B	2	0.007	0.6	0.6	ND	11	0.1
A036D	20	5.6	56	0.4	ND	22	>30
P022A	1	0.03	0.4	0.5	1.6	32	0.3
P022C	16	5.6	40	0.5	11	32	>30
P026A	0	0.01	0.4	0.4	2	28	0.3
P026B	11	2.8	100	0.1	2.5	10	>30

(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. Four of the 11 patients had no detectable p24 antigen throughout the study and two of the three patients who died had no detectable antigen for many months prior to death. 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

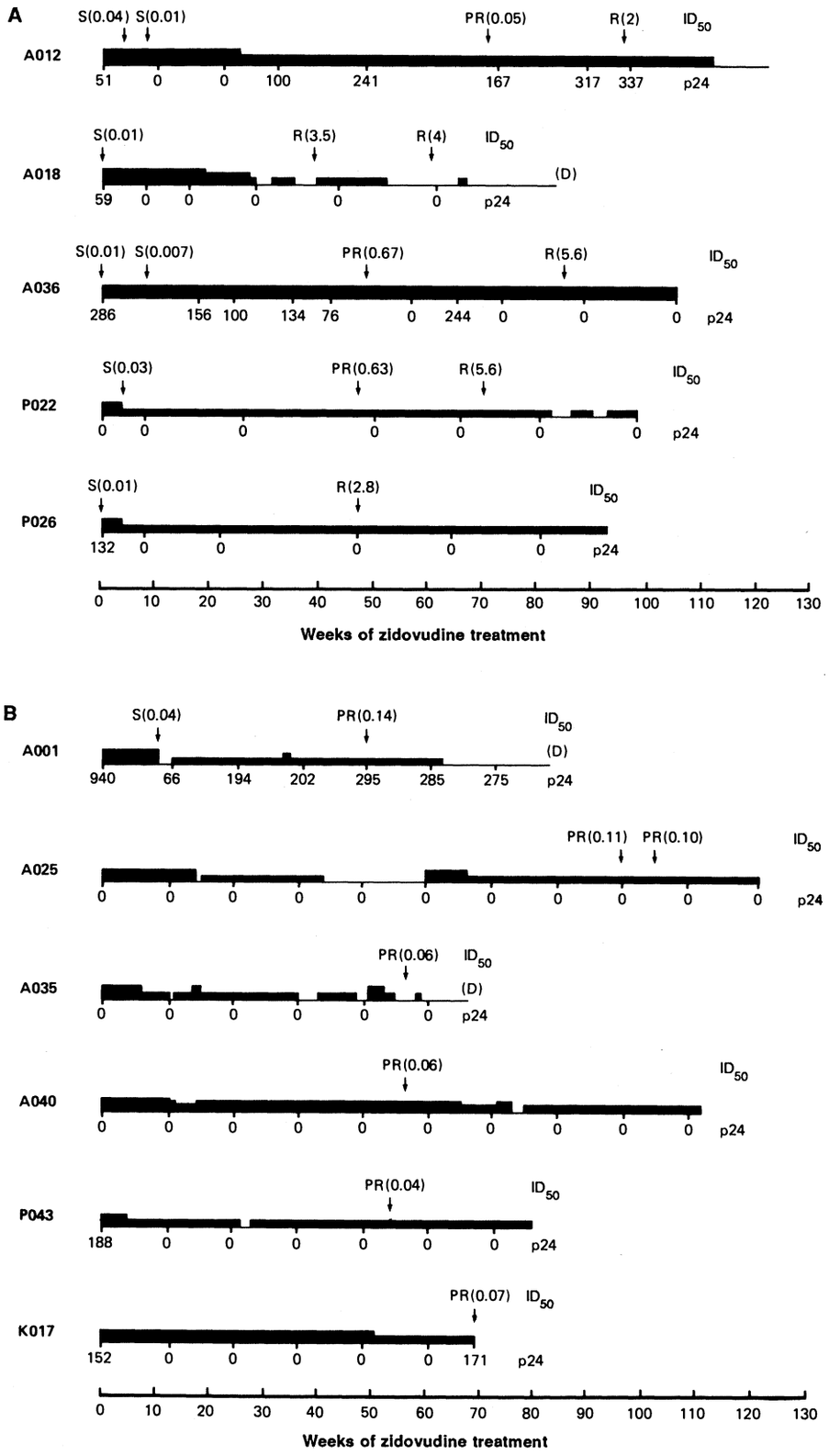

Fig. 3. Therapy and disease patterns of patients treated for more than 6 months. The oral dose of zidovudine indicated by the horizontal bar was either 250 mg (broad bar), 200 mg (intermediate), or 100 mg (narrow bar) given every 4 hours. The viruses isolated are classified either S (ID_{50} < 0.05 μM, ID_{95} < 1 μM), R (ID_{50} > 1 μM), or PR (all other isolates). ID_{50} 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, medium 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, medium of 80 (range 80–90); and body weight, 65.3 kg (range 54–81 kg).

therefore be of limited value.

A further question relates to the virulence 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.

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14. Each virus was propagated in MT-4 cells (*9*) and clarified infected cell supernatants were prepared from cultures at peak p24 antigen levels (in excess of 10^5 pg/ml). Virus was precipitated from these supernatants at 4°C with polyethylene glycol (10%) and NaCl (0.1*M*). Pellets were washed with cold phosphate-buffered saline and virus was solubilized in buffer containing 0.5% triton X-100, 500 m*M* KCl, 50 m*M* tris-HCl *p*H 7.5, 1 m*M* phenylmethylsulfonyl fluoride, and 5 m*M* β-mercaptoethanol. RT activity was assayed with poly(rA)·oligo(dT) as primer template and [^{3}H]TTP (5 μ*M* and 10 μCi/ml) as described [B. A. Larder, D. J. M. Purifoy, K. L. Powell, G. Darby, *EMBO J.* **6**, 3133 (1987)] and inhibition experiments with zidovudine triphosphate were performed as described (*13*).
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