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Effects of Discontinuation of Zidovudine Treatment on Zidovudine Sensitivity of Human Immunodeficiency Virus Type 1 Isolates

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Zidovudine treatment of individuals infected with human immunodeficiency virus type 1 (HIV-1) results in HIV-1 isolates with a reduced zidovudine sensitivity in vitro. This reduction is due to mutations causing amino acid substitutions at five codons (41, 67, 70, 215, and 219) on the reverse transcriptase enzyme of HIV. HIV-1 isolates were obtained 8 to 69 weeks after therapy discontinuation from 10 patients at different stages of disease. Zidovudine sensitivity was determined by the HeLa CD4$^+$ plaque assay. The presence of the resistance-conferring mutations was determined by using a selective polymerase chain reaction. Sensitivity could be determined for six isolate pairs: one showed a decline in the 50% inhibitory zidovudine concentration after therapy discontinuation; four pairs did not show a change. The majority of changes in the five codons in isolates from all 10 patients were the result of a relative increase in the wild-type sequence. Complete changes from mutant to the wild type were seen for only two codons in isolates from two patients. This study of isolates from a small group of individuals at different stages of disease, who had been taking zidovudine for 1 to 2 years, shows that a period of 1 year without zidovudine may be required to achieve a change from a mutant or mixed virus population to a wild-type virus population.

During zidovudine treatment of human immunodeficiency virus type 1 (HIV-1)-infected individuals, HIV isolates with a reduced zidovudine sensitivity in vitro are generated (10). Reduction in sensitivity is caused by mutations resulting in amino acid substitutions at five positions (41, 67, 70, 215, and 219) on the reverse transcriptase (RT) enzyme of HIV (6, 11, 12). These mutations appeared in an ordered fashion during treatment of a cohort of asymptomatic individuals (2). The first mutation appears after several weeks of treatment transiently at codon 70 (Lys to Arg); its disappearance within several months is paralleled by the appearance of two linked mutations resulting in an amino acid change at codon 215 (Thr to Tyr or Phe) and followed shortly thereafter by a mutation at codon 41 (Met to Leu) (6). Subsequently, the codon 70 mutation reappears, probably in most cases in parallel with the mutation at codon 67 (Asp to Asn). Finally, the mutation causing the amino acid change at codon 219 (Lys to Gln) may appear. For some patients, however, no change at codon 41 is found. A similar pattern was observed during treatment of a group including both symptomatic and asymptomatic individuals (13). In vitro serial passage of the HIV laboratory strain HXB-2 in the presence of gradually increasing concentrations of zidovudine gives a similar order of appearance of mutations (9).

The effects of the amino acid changes on zidovudine sensitivity were studied by introduction of the encoding mutations in the molecular clone HXB-2 (6, 11, 12). Single amino acid changes at codons 41, 70, and 215 result in 4- to 16-fold decreases in sensitivity compared with wild-type viruses. Addition of the codon 41 change to the codon 215 change creates a variant with a 60-fold decrease in sensitivity compared with that of the wild-type variant. A further decrease can be achieved by adding the amino acid changes at codons 70 and 67 in combination with the codon 41 and 215 mutations (approximately 180-fold compared with the wild type). However, some clinical isolates with mutations at codons 67, 70, and 215 have a mutation at codon 219 instead of 41; this combination results in an approximately 120-fold decrease in sensitivity. Comparison of the sensitivity obtained with molecular clones containing these mutations with the sensitivity of clinical isolates with an identical genotype shows that the level of sensitivity can be predicted in most cases by analysis for these five mutations (6).

We studied the effect of viral replication in the absence of zidovudine both in vitro and in vivo. The stabilities of the mutations causing reduced zidovudine sensitivity were investigated by serial passages of several molecular clones possessing either one mutation or a combination of mutations. To determine the difference in replicative capacity, a mixture of wild-type virus with virus containing an amino acid change at position 215 was passaged in the absence of the drug. The in vivo effect of treatment discontinuation was analyzed by studying primary HIV isolates longitudinally obtained from individuals who had to discontinue treatment because of hematologic toxicity. These isolates were tested both for their biological sensitivities and for the presence of the described mutations.

MATERIALS AND METHODS

Study population. Isolates were obtained from 10 homosexual men who discontinued zidovudine therapy because of hematologic toxicity due to the drug. Four patients (D, E, F,

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and J) participated in a previously described zidovudine dose efficacy study (3). Six patients were from the AIDS clinic of the Academic Medical Center in Amsterdam, The Netherlands. Patient H used alpha interferon as an antiviral comedication. The clinical statuses of these individuals were defined according to the criteria of the Centers for Disease Control (4). $CD4^+$ lymphocytes were enumerated by direct immunofluorescence using monoclonal antibodies (Central Laboratory of The Netherlands, Red Cross Blood Transfusion Service, Amsterdam, and Becton Dickinson, Mountain View, Calif.) and a flow cytometry system (EPICS-C; Coulter, Luton, United Kingdom). The level of HIV-1 p24 antigen in serum was determined by using a solid-phase sandwich-type immunoassay (Abbott Laboratories, Chicago, Ill.) as described elsewhere (5).

Changes in CD4 counts and p24 antigen levels associated with the stop of zidovudine therapy were calculated by using the mean of two consecutive values within a 24-week period before the moment of discontinuation of zidovudine treatment and of two consecutive values after this moment (when no second sample was available, one value was used).

HIV-1 culture. HIV was isolated by cocultivating 10^6 cryopreserved peripheral blood mononuclear cells (PBMC), obtained by density gradient centrifugation on Ficoll-Hypaque, with 5×10^6 phytohemagglutinin-stimulated peripheral blood lymphocytes (PBL) from an HIV antibody-negative blood donor. Twice a week, cultures were screened for the appearance of syncytia and culture supernatants were tested for the presence of HIV p24 antigen (Abbott Laboratories). Supernatants or cells from PBMC-PBL cocultures were cultured with 5×10^6 cells from a T-lymphoblastoid cell line (MT-2) in order to obtain high-titer virus stocks. These supernatants were used for drug sensitivity testing.

Zidovudine sensitivity. Plaque reduction assays were performed in duplicate by infection of HeLa $CD4^+$ (HT4 LacZ) cell monolayers as previously described (8). The 50 and 90% inhibitory concentrations (IC_{50} and IC_{90}, respectively) of micromolar zidovudine were derived from plots of the mean percent inhibition versus zidovudine concentration. On the basis of IC_{50}, viral isolates can be divided into three groups: sensitive ($IC_{50} < 0.01$ μM), partly resistant (0.01 μM < IC_{50} < 1.0 μM), and highly resistant (>100-fold increase or IC_{50} > 1.0 μM). On the basis of results obtained through repetitive testing of isolates derived from molecular clones with various combinations of mutations, a threefold or greater change in IC_{50} was considered significant. If a significant decrease for a patient over time was observed, viral stocks were retested.

Passage of molecular clones in the absence of zidovudine. Variants constructed by site-directed mutagenesis (11, 12) containing mutations in the RT gene were used. Homogeneous inocula of HXB-2 and the constructs HIV-RTMF (Thr-215 → Tyr), HIV-RTMJ (Lys-70 → Arg), and HIV-RTMC (Asp-67 → Asn, Lys-70 → Arg, Thr-215 → Phe, and Lys-219 → Gln), derived from HXB-2, were passed in the absence of zidovudine. In addition, a mixture consisting of equal numbers of plaque-forming viruses of HIV-RTMF (Tyr-215) and HXB-2 was passaged in the absence of zidovudine.

Cell-free inocula were used to infect 5 million MT-2 cells; supernatants were harvested when syncytia were observed in every cell clump. A proportion of the supernatant was used to passage cell-free virus to infect new MT-2 cells; another proportion was stored at −70°C. Supernatants were tested in parallel by the HeLa $CD4^+$ plaque assay.

Genotypic analysis. A selective polymerase chain reaction (PCR) was used for analysis of changes in the five codons (41, 67, 70, 215, and 219) of the RT gene causing zidovudine resistance. Provirus DNA extracted from approximately 10^6 cocultivated PBMC-PBL was amplified by a nested procedure (3, 11). Initially, in a first amplification procedure a region of the provirus RT gene containing all mutations was amplified. Subsequently, in order to discriminate between wild-type and mutant codons, a second selective procedure was performed for each codon by using one primer to detect the wild type and another primer to detect mutant virus in two separate PCRs. The PCR products were analyzed and scored as described previously (3).

RESULTS

Zidovudine sensitivity. Paired HIV isolates were obtained from 10 patients through cocultivation of the patients' PBMC and donor PBL (Table 1). For each patient, a baseline isolate was obtained either before or at therapy discontinuation (for nine patients) or shortly thereafter (2 weeks for patient A). Posttherapy isolates were obtained 8 to 69 weeks after therapy discontinuation. Paired isolates from six patients were compared for zidovudine sensitivity, because both the baseline and the posttherapy isolates were MT-2 cell tropic and formed plaques in the HeLa $CD4^+$ plaque assay. Only one patient (A) had a highly resistant population when therapy stopped (IC_{50}, 5.2 μM), and for this patient 8 weeks later no significant change was observed. Five patients had partly resistant isolates (IC_{50}, 0.01 to 0.50 μM) at therapy discontinuation. Isolates from three patients (B, D, and E) did not show a significant change in IC_{50} after 13, 20, or 32 weeks without therapy. For one patient (H), an approximately 30-fold decline in IC_{50} was measured after 8 weeks without therapy. Remarkably, for one patient (I) a fourfold decline in IC_{50} was observed, resulting in a sensitive isolate, while according to the information obtained from the patient he was still taking zidovudine.

Genotypic analysis. Provirus DNAs from primary isolates generated by cocultivation of patient PBMC and donor PBL were analyzed by using the selective PCR approach for the composition of five amino acids.

(i) Codon 41. Paired isolates from seven patients were analyzed for composition at codon 41. In isolates from four patients (B, D, E, and H) a mutant population was initially observed; for patient D the mutant population persisted for 20 weeks, for patient B a change to a mixed population had occurred after 13 weeks, for patient E a change to a mixed population was observed after 32 weeks, and for patient H a change to a wild-type population was found after 59 weeks. In isolates from three patients (A, G, and I), around the time therapy stopped a mixed population was present. For patient A, no change was seen after 8 weeks. A change to a wild-type population was detected at week 13 for patient I and at week 29 for patient G.

(ii) Codon 67. Only one patient (A) had a mutant population at the initial sampling; 6 weeks later, a mixed population was present. In patient H, a mixed population persisted for 59 weeks. A change from a mixed to a wild-type population in patient I was observed during therapy. Six other patients had wild-type codons when therapy stopped, and the codons remained wild type.

(iii) Codon 70. Isolates obtained from two patients (A and K) during therapy were mutant; in patient A the mutant population persisted for 8 weeks, and the virus population from patient K was wild type after 69 weeks. Isolates from two patients (B and H) consisted of a mixed population; for

TABLE 1. Patient profile

Patient	CDC stage (CD4 count)[a]	Time (wk) of sample[b]	RT at codon[c]:					Zidovudine			
								IC[d]		Previous treatment	
			41	67	70	215	219	50%	90%	Wk	mg/day
A	IV-C1 (10)	2	Mix	Mut	Mut	Mut	Mix	5.2	>10	15	1,200
		8	Mix	Mix	Mut	Mut	Mix	3.6	>10	2	600
										27	1,000/1,200
										3	0
										9	600
B	IV-C1 (16)	−2	Mut	WT	Mix	Mut	WT	0.20	>10	54	600
		13	Mix	WT	Mix	Mut	WT	0.25	>10	5	0
										15	600
D	II/III (40)	−4	Mut	WT	WT	Mut	WT	0.16	>10	59	1,000
		20	Mut	WT	WT	Mut	WT	0.19	>10	51	800
										17	600
										16	400
E	IV-C2 (60)	−30	Mut	WT	WT	Mut	WT	0.50 (0.35)	8.2	59	1,000
		32	Mix	WT	WT	Mut	WT	0.16 (0.38)	>10	10	500
										17	600
										10	400
F	II/III (100)	−8	Mut	WT	Mut	Mut	WT	0.50	>2	4	2,000
	IV-C2	32	—	—	—	Mut	—	—	—	118	1,000
G	IV-C1 (20)	−13	Mix	WT	WT	Mut	WT	—	—	77	1,000/1,200
		18	Mix	WT	WT	Mut	WT	—	—		
		29	WT	WT	Mut	Mix	WT	0.005	0.09		
H	IV-D (20)	0	Mut	Mix	Mix	Mut	WT	0.29 (0.56)	>10	55	1,000
		8	Mut	Mix	Mix	Mut	WT	0.01 (0.01)	>10	37	600/400
		59	WT	Mix	Mix	Mix	WT	0.01 (0.01)	0.25		
I	IV-C2 (20)	−22	WT	Mix	Mut	Mix	WT	0.02 (0.02)	>2	30	1,200
		−4	Mix	WT	Mix	WT	WT	0.005	0.72	31	600/500
		13	WT	WT	—	WT	—	0.005 (0.007)	0.28		
J	II/III (200)	−15	Mut	WT	WT	Mut	WT	—	—	112[e]	1,000
		52	—	WT	WT	Mut	WT	—	—		
K	IV-A (100)	−1	—	WT	Mut	WT	WT	—	—	22	1,000
		69	—	WT	WT	WT	WT	—	—	4	600
										15	0
										13	1,000
										4	500

[a] CDC, Centers for Disease Control (4). The CD4 counts were determined around the time of therapy discontinuation.
[b] Before (−) or after therapy discontinuation.
[c] Results of PCR analysis (9). Mix, mixed; Mut, mutant; WT, wild type; —, not tested.
[d] As determined by the HeLa CD4$^+$ plaque assay (8). The result of a second independent determination is given in parentheses. —, not determined.
[e] With some interruptions.

patient B the mixed population was still present after 13 weeks, and for patient H a mixed population was still found after 59 weeks. Isolates obtained from four patients during therapy were wild type at codon 70; one of the patients (G) developed a mutation in the absence of the drug.

(iv) **Codon 215.** Eight isolates obtained initially were mutant. The mutant populations persisted in five patients: 8 weeks in patient A, 13 weeks in patient B, 20 weeks in patient D, 32 weeks in patients E and F, and 52 weeks in patient J. A change from a predominantly mutant to a mixed population was observed for two patients: patient G at 29 weeks and patient H at 59 weeks. A mixed population was observed 22 weeks before drug discontinuation for patient I, and after 18 weeks of therapy it had changed to a wild-type population.

(v) **Codon 219.** Only one individual (A) harbored a mixed population, which persisted for 8 weeks without zidovudine; all other patients had wild-type populations throughout.

Passage of molecular clones in the absence of zidovudine. The sensitive wild-type reference strain HXB-2 and three molecular clones, two partly resistant (HIV-RTMJ [Arg at codon 70] and HIV-RTMF [Tyr at codon 215]) and one highly resistant (HIV-RTMC), were propagated in the absence of zidovudine. The IC_{50} and IC_{90} for passages at 0, 6, and 12 weeks did not show significant changes during this period (except for a sixfold increase in IC_{90} during 12 passages for HIV-RTMF [data not shown]), which was in contrast to the results obtained when an equal mixture of HXB-2 with HIV-RTMF was passaged. An increase in the proportion of sensitive viruses was observed after 20 pas-

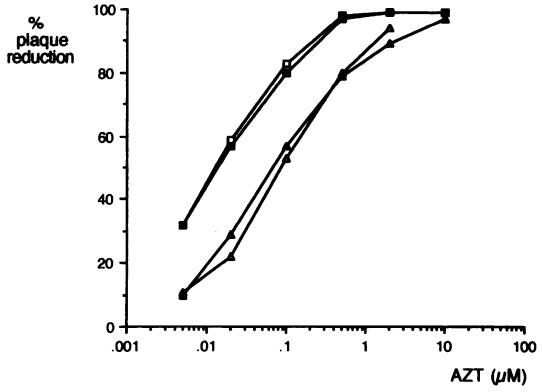

FIG. 1. A mixture of equal amounts of sensitive, wild-type HIV HXB-2 (threonine at codon 215) and partly resistant HIV-RTMF (tyrosine at codon 215) was passaged in the absence of zidovudine (AZT). Zidovudine sensitivity was tested (in duplicate) at passage 1 (△ and ▲) and passage 20 (□ and ■) by the HeLa CD4$^+$ plaque assay.

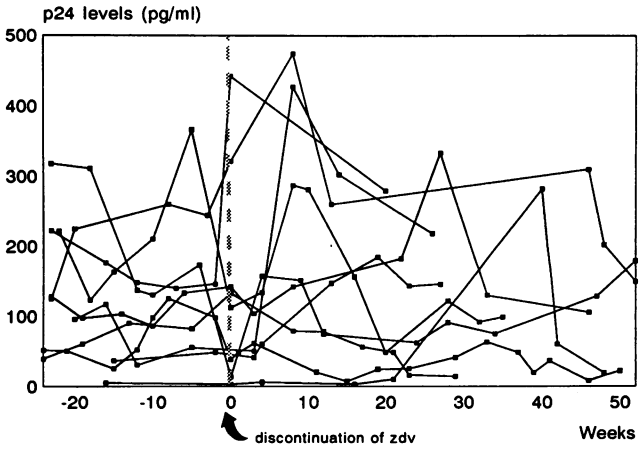

FIG. 3. The effects of permanent discontinuation of zidovudine (zdv) treatment on the HIV p24 antigen levels. Each line represents one patient.

sages (Fig. 1). Sevenfold decreases in IC_{50} and IC_{90} were observed.

Changes in surrogate markers after discontinuation of zidovudine treatment. CD4 counts and p24 antigen levels were available for all patients for at least one visit before permanent discontinuation of zidovudine and for at least one later visit (except for the CD4 counts of patient K).

The changes in CD4 counts after discontinuation of zidovudine treatment are indicated in Fig. 2. The posttreatment CD4 counts dropped in seven patients after zidovudine treatment was stopped and remained stable in two patients, who both had CD4 counts of 10 cells per mm^3. In none of the patients was a rise in CD4 counts seen. Overall, significant drops in CD4 counts after the discontinuation of zidovudine were observed; the mean decrease from pretreatment values was 50% (95% confidence interval, 16.9 to 67.3%).

The changes in the p24 antigen levels are shown in Fig. 3. The posttreatment p24 antigen levels in four patients had dropped, while an increase was seen for six patients. The overall change in serum p24 values was not significant, with the mean increase from pretreatment values being 59% (95% confidence interval, −47.8 to 165.0%). No correlation between changes in CD4 cell counts and changes p24 antigen levels was found; among patients whose CD4 counts had dropped after discontinuation of zidovudine treatment, increases in the levels of p24 antigen were observed for four patients while decreases were observed for three patients.

One patient (J) showed a rise in the CD4 count and a drop in the p24 antigen level 20 weeks after discontinuation of treatment. Since these changes were paralleled by an increase in the erythrocyte mean capsular volume, it cannot be excluded that this patient had been taken zidovudine for some period despite the fact that drug discontinuation was recommended by his physician.

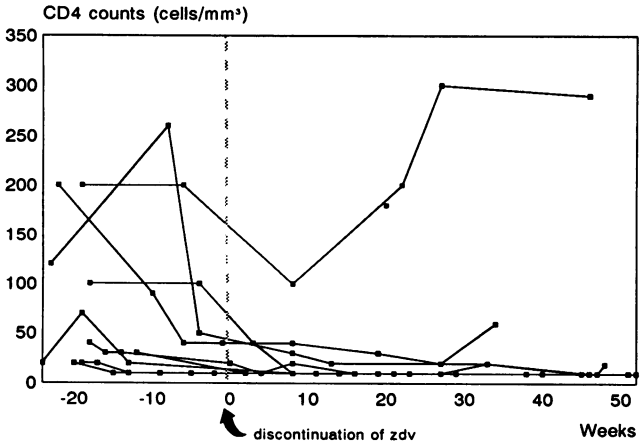

FIG. 2. The effects of permanent discontinuation of zidovudine (zdv) treatment on CD4 counts. Each line represents one patient.

DISCUSSION

We studied the changes in zidovudine sensitivity and presence of mutations in six paired clinical HIV isolates, with each pair consisting of one isolate obtained around the time of therapy cessation and another obtained later. The presence of zidovudine resistance-conferring mutations was studied with these six pairs and four additional pairs. One patient had highly resistant isolates, and five patients had partly resistant isolates at the time of therapy discontinuation. In five virus pairs no significant decrease in IC_{50} or IC_{90} was observed after 8 to 32 weeks. For one patient with a partly resistant isolate, decreases in IC_{50} and IC_{90} were measured; a fully sensitive population was present after 59 weeks without treatment. An unanticipated change was seen for patient I; a decline in resistance in this patient was observed while, according to the information given by the patient, he was still on zidovudine therapy. It may be that this information was incorrect.

The effects of both individual mutations and combinations on zidovudine sensitivity in clinical isolates were recently clarified by the introduction of these mutations in the laboratory HIV strain HXB-2 (6, 11, 12). In this study, the composition of the five codons from primary isolates was analyzed by a selective PCR approach. This approach enables us to distinguish three sorts of populations for each codon: wild type, mixed, and mutant. The IC_{50} for the clinical isolates in this study were within the range predicted by the genetic analysis. An exception was the isolate from patient H, obtained 8 weeks after therapy discontinuation.

This isolate consisted of a population with homogenous mutations both at codon 215 and at codon 41 and a mixed population at codons 67 and 70. However, the IC_{50} of 0.01 μM for this clinical isolate was 60-fold lower than expected (6). Therefore, we analyzed provirus DNA from this sample obtained from the HeLa CD4 cells used for the biological-sensitivity testing. Provirus DNA from the HeLa CD4 cells and provirus DNA from the cocultivated PBMC contained similar patterns of mutations. Thus, selection of a viral population during viral-stock preparation or during sensitivity testing can be excluded. The RT gene of this isolate will be sequenced to determine whether the virus acquired an additional mutation which reduces the effect of zidovudine resistance (14).

The majority of changes in the five codons over time in this patient group resulted in a relative increase in the wild-type sequence. A change from a mutant to a wild-type population was seen in only two instances: for one patient with a mutation at codon 70 and another patient with a mutation at codon 41. All other changes were partial, either from a mutant to a mixed population or from a mixed to a wild-type population.

The results of reconstruction experiments with DNA obtained from wild-type HXB-2 (Thr-215) and mutant HIV-RTMF (Tyr-215) indicate that in patients in whom mixed populations persist over time, it is possible that considerable changes in the wild-type-to-mutant virus ratio occur. An increase in the wild-type sequence after discontinuation of therapy can be caused by one of two mechanisms: molecular reversion of the mutation to the wild type or selective outgrowth of the wild-type virus. Propagation in vitro of homogeneous partly resistant populations containing either the codon 70 or the codon 215 change or a highly resistant population possessing a combination of four mutations (at codons 67, 70, 215, and 219) in the absence of zidovudine does not result in a change in the viral sensitivity profiles. This indicates that molecular reversal does not occur easily. Propagation of a population consisting of a mixture of wild-type, sensitive virus and partly resistant virus containing a codon 215 change caused an increase in the proportion of wild-type virus. Thus, laboratory strains possessing RT mutations might have a slight in vitro growth disadvantage, probably caused by small changes in their replicative capacities.

One of the hallmarks of retrovirus infection is persistence of integrated provirus DNA. The time required for a detectable change in viral population, if caused by a difference in replication rate, will depend on two factors: the persistence of proviruses, which is determined by the life span of the host cell, and the mutant-to-wild-type sequence ratio at the time of therapy withdrawal. This ratio will depend on treatment duration (2, 3). Thus, the time required for a detectable change after treatment discontinuation may vary with the biological characteristics of the virus and the duration of treatment.

St. Clair et al. (14) analyzed single clones of HIV isolates of several individuals who changed from zidovudine treatment to 2′,3′-dideoxyinosine. In these individuals, a change towards the wild type was noted for codons 41 and 70 after several months of zidovudine withdrawal. In contrast, codon 215 mutations persisted for almost a year, even in the presence of a mutation at codon 74 causing 2′,3′-dideoxyinosine resistance. Land et al. (7) reported a change to sensitive virus in some patients for whom zidovudine therapy had to be stopped and persistence of resistant virus in others. Albert et al. (1) reported the appearance of wild-type sequences after discontinuation of therapy.

In some instances, we found a homogeneous mutant population and no wild-type virus at the time of therapy withdrawal and a subsequent change to wild-type codons. In this case, it is unlikely that the change to the wild type is caused by the outgrowth of a minority population of wild-type virus present in the PBMC. Possible mechanisms in these instances are molecular reversal or outgrowth of wild-type viruses not residing in the peripheral circulating cells.

The drops in CD4 counts in seven of nine patients after zidovudine treatment was stopped suggest that some patients may have benefited from this drug, even though their isolates showed at least some degree of genotypic or phenotypic resistance at this point. Alternatively, one might argue that, since no control group is available, it is not known whether the prolongation of zidovudine treatment beyond this point would have prevented the drops in CD4 counts. The increases in the p24 antigen values in six patients after discontinuation of treatment also suggest that some benefit of zidovudine may be present in patients with resistant isolates. However, p24 antigen levels did not change in a consistent fashion after the discontinuation of zidovudine treatment, and it may therefore be an unreliable marker for viral replication in this situation. A control group of patients for whom zidovudine treatment is continued would be necessary to evaluate the actual benefit of zidovudine at this point.

In conclusion, this study of a relatively small group of individuals at different stages of disease ranging from asymptomatic to full-blown AIDS, who had been taking zidovudine for 1 to 2 years, shows that a change from mutant or mixed isolates to fully wild-type isolates does not occur rapidly and may depend on the duration of the treatment.

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REFERENCES

1. **Albert, J., J. Wahlberg, J. Lundeberg, S. Cox, E. Sandström, B. Wahren, and M. Uhlen.** 1992. Persistence of azidothymidine-resistant human immunodeficiency virus type 1 RNA genotypes in posttreatment sera. J. Virol. **66**:5627–5630.
2. **Boucher, C. A. B., E. O'Sullivan, J. W. Mulder, C. Ramautarsing, P. Kellam, G. Darby, J. M. A. Lange, J. Goudsmit, and B. A. Larder.** 1992. Ordered appearance of zidovudine (AZT) resistance mutations during treatment. J. Infect. Dis. **165**:105–110.
3. **Boucher, C. A. B., M. Tersmette, J. M. A. Lange, P. Kellam, R. E. Y. de Goede, J. W. Mulder, G. Darby, J. Goudsmit, and B. A. Larder.** 1990. Zidovudine sensitivity of human immunodeficiency viruses from high-risk, symptom-free individuals during therapy. Lancet **336**:585–590.
4. **Centers for Disease Control.** 1986. Classification system for human T lymphotropic virus type III/lymphadenopathy-associated virus infections. Morbid. Mortal. Weekly Rep. **35**:334–339.
5. **Goudsmit, J., F. de Wolf, D. A. Paul, L. G. Epstein, J. M. A. Lange, W. J. A. Krone, H. Speelman, E. C. Wolters, J. van der Noordaa, J. M. Oleske, H. J. van der Helm, and R. A. Coutinho.** 1986. Expression of human immunodeficiency virus antigen (HIV-Ag) in serum and cerebrospinal fluid during acute and chronic infection. Lancet **ii**:177–180.
6. **Kellam, P., C. A. B. Boucher, and B. A. Larder.** 1992. Fifth

mutation in HIV-1 reverse transcriptase contributes to the development of high level resistance to zidovudine. Proc. Natl. Acad. Sci. USA **89:**1934–1938.

7. **Land, S., K. McGavin, C. Birch, and R. Lucas.** 1991. Reversion from zidovudine resistance to sensitivity on cessation of treatment. Lancet **338:**830–831.
8. **Larder, B. A., B. Chesebro, and D. D. Richman.** 1990. Susceptibilities of zidovudine-susceptible and -resistant human immunodeficiency virus isolates to antiviral agents determined by using a quantitative plaque reduction assay. Antimicrob. Agents Chemother. **34:**436–441.
9. **Larder, B. A., K. E. Coates, and S. D. Kemp.** 1991. Zidovudine-resistant human immunodeficiency virus selected by passage in cell culture. J. Virol. **65:**5232–5236.
10. **Larder, B. A., G. Darby, and D. D. Richman.** 1989. HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science **243:**1731–1734.
11. **Larder, B. A., P. Kellam, and S. D. Kemp.** 1991. Zidovudine resistance predicted by direct detection of mutations in DNA from HIV-infected lymphocytes. AIDS **5:**137–144.
12. **Larder, B. A., and S. D. Kemp.** 1989. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). Science **246:**1155–1158.
13. **Richman, D. D., J. C. Guatelli, J. Grimes, A. Tsiatis, and T. Gingeras.** 1991. Detection of mutations associated with zidovudine resistance in human immunodeficiency virus by use of the polymerase chain reaction. J. Infect. Dis. **164:**1075–1081.
14. **St. Clair, M. H., J. L. Martin, G. Tudor-Williams, M. C. Bach, C. L. Vavro, D. M. King, P. Kellam, S. D. Kemp, and B. A. Larder.** 1991. Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. Science **253:**1557–1559.