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Reverse Transcriptase Sequence of Paired Isolates of Cerebrospinal Fluid and Blood from Patients Infected with Human Immunodeficiency Virus Type 1 during Zidovudine Treatment

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Human immunodeficiency virus type 1 (HIV-1) isolates obtained from the blood of patients undergoing treatment with 3′-azido-3′-deoxythymidine (zidovudine [AZT]) show a decreased sensitivity to the drug in vitro. The aim of the present study was to determine if HIV-1 variants resistant to AZT are present also in the brain compartment. We selected sequential HIV-1 isolates from the blood and the cerebrospinal fluid (CSF) of six patients with HIV-1 infection undergoing AZT therapy for a time varying between 1 and 3 years. The isolates were used to infect peripheral blood mononuclear cell cultures which were used to prepare viral DNA. The viral DNA was amplified by PCR and then directly sequenced. Analysis of the reverse transcriptase (RT) sequence of the isolates from the CSF during therapy demonstrated that CSF-resistant isolates are characterized by the same mutations documented in resistant isolates from the blood compartment. Isolates obtained from one patient (patient 3) showed the same two mutations (codons 70 and 215) in blood and CSF, whereas isolates obtained from an additional four patients presented a different pattern of mutations in the two compartments. We also analyzed the degree of amino acid homology between RT sequences from blood and CSF isolates in patients before and during AZT treatment. The percentages of amino acid variations were approximately equal when isolates from the same or different compartments were considered. Excluding the codons involved in AZT resistance, the time point of sampling did not affect RT variations during therapy significantly. In conclusion, our studies show that AZT-resistant HIV-1 can be found in the CSF of patients undergoing treatment. The mutations linked to AZT resistance in the CSF isolates are the same as those identified in AZT-resistant isolates from blood.

AIDS is often accompanied by a variety of neurological manifestations which mostly appear in the terminal phase of the disease. Infection of the nervous system by human immunodeficiency virus type 1 (HIV-1) is likely to be established by infected macrophages which cross the blood-brain barrier. Virus replication in the brain occurs in the microglia cells (5, 6), which have been shown to be the main target of HIV-1 infection in this compartment (12, 26). The CD4 receptor present on the surface of microglia cells mediates HIV-1 infection (9).

The pathogenic mechanism(s) which leads to the development of neurological syndromes during AIDS is not well characterized. Whether virus variants with marked tropism for brain cells are involved in the process of infection of the brain is still an open question. Studies on the nucleotide sequences of variable region 3 (V3 loop) of the gp120 envelope protein have shown that strains isolated from cerebrospinal fluid (CSF) and blood from patients in the advanced stage of disease differ from one another (10). These differences may be due to mechanisms of adaptation or immunomodulation of the virus in the two compartments during the course of the disease, as indicated by the high degree of conservation of V3 sequences in the two different organs during the early stage of disease (10).

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The study of viral proteins that are not the targets of protective immunity and do not participate in the events of virus tropism will likely help us to understand the mechanisms responsible for virus diversification over time and intertissue. Zidovudine (azidothymidine [AZT]) has been shown to improve $CD4^+$ cell count, to reduce HIV-1 p24 concentration in plasma, to decrease the frequency of opportunistic infection, and to prolong survival (19). There is clear evidence of a therapeutic temporal effect of this drug on patients with AIDS neurological syndromes (23). Prolonged clinical use of AZT leads to the emergence of resistant HIV-1 isolates in the blood compartment (3, 17, 18). The resistance genotype is characterized by five amino acid substitutions which appear alone or in combination at codons 41, 67, 70, 215, and 219 of the reverse transcriptase (RT) gene.

The aim of the present study was to sequence the RT gene of HIV-1 isolates from the CSF and blood in order to estimate the degree of variation between the two compartments over time. We also intended to verify whether isolates from the CSF became resistant to AZT and if they were characterized by the same mutations present in the blood.

MATERIALS AND METHODS

Virus isolates. Five patients with HIV-1 infection in Centers for Disease Control and Prevention (CDC) stage III and one patient in stage IV were enrolled in this study. Patient 1 (CDC IV) showed neurological symptoms associated with HIV-1 infection. All patients were part of an ongoing prospective study which included lumbar puncture. Routine parameters form CSF analysis

TABLE 1. Clinical routine and AZT IC_{50} determinations in six HIV-1-infected patients

Patient	Mo of therapy	AZT (mg/day)	CD4 count per liter (10^9)	IC_{50} (μmol/liter)		CSF analysis	
				CSF	Blood	Cells (10^6)[a]	Protein (g/liter)[b]
1	0[c]	500	0.21	0.08	0.07	10	0.37
	13		0.04	8.2	8.7	0	0.37
2	0	400	0.46	0.03	0.3	6	0.47
	29		0.11	0.4	11	1	0.42
3	0	1,200	0.3	0.15	0.32	1	0.44
	38		0.16	5	6.8	1	0.38
4	0	500	0.52	0.06	0.005	11	0.42
	10		ND[d]	0.4	0.38	1	0.40
5	0	500	0.41	0.015	0.065	6	0.91
	18		0.48	6	0.3	3	0.43
6	0	400	0.28	0.3	0.015	1	0.38
	24		0.38	1.85	2.5	2	0.42

[a] Normal value, $<5 \times 10^6$/liter.
[b] Normal value <0.7 g/liter.
[c] 0 = start point of AZT treatment.
[d] ND, not determined.

are shown in Table 1. Glucose levels were normal in all CSF specimens. The patients had received AZT treatment for periods of time varying between 1 and 3 years. The $CD4^+$ cell count at enrollment varied between 0.21×10^9 and 0.52×10^9/liter (Table 1).

Sequential HIV-1 isolates from blood and CSF were obtained by cocultivation of patient samples with phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMCs) from healthy seronegative blood donors, as previously described (2). The isolates from the blood were obtained from patient PBMCs, and the CSF strains were from inoculation of both cells and fluid. In order to obtain virus stocks, the isolates were passaged once onto phytohemagglutinin-stimulated PBMCs from seronegative blood donors.

AZT sensitivity of HIV-1 isolates. The HIV-1 isolates were tested for sensitivity to AZT by monitoring the inhibition of p24 production in PBMC cultures treated with scalar doses of AZT. The isolates from each culture were assayed in parallel, and 100 50% tissue culture infective doses from each patient virus strain were used. The strains were divided into sensitive and partly and highly resistant phenotypes on the basis of the 50% inhibiting concentration of AZT (IC_{50}). Viral isolates with an IC_{50} of 0.3 μmol/liter or less were considered sensitive, those with an IC_{50} between 0.3 and 1 μmol/liter were partly resistant, and those with an IC_{50} >1.0 μmol/liter were highly resistant. All determinations included an AZT-sensitive reference strain with a known IC_{50}. Repeated and independent analyses of the reference HIV-1 strain showed a within-assay variation of less than 30% (15).

Solid-phase DNA sequencing of RT fragments. PBMCs, infected with the same virus passage used to characterize the biological phenotype, were used to prepare DNA samples for PCR. The cells were washed twice in phosphate-buffered saline and then lysed in 0.1 ml of PCR lysing buffer (10 mM Tris-HCl [pH 8.3], 1 mM EDTA, 0.5% Nonidet P-40, 0.5% Tween 20, 0.3 mg of proteinase K per ml) at 65°C for 30 min. Proteinase K was inactivated by incubating the samples at 95°C for 15 min.

PCR was performed in two-step reactions, as previously reported (1). In the first amplification, a fragment of 930 bp was obtained with the following oligonucleotide primers: JA99, 5′-GGG GGA ATT GGA GGT TTA TCA AAG-3′ (strain 310-335 MN [22]); and JA4, 5′-TTC TGT ATG TCA TTG ACA GTC CAG C-3′ (strain 1240-1216 MN). PCR was done in 30 cycles, each consisting of denaturation at 94°C for 30 min, annealing at 60°C for 30 min, and elongation at 74°C for 1 min, plus a final extention at 74°C for 10 min. An additional 30 cycles were conducted with the inner primers JA100 (5′-GAC CTA CAC CTG TCA ACA TAA TTG G-3′ [strain 401-425 MN] and M3 (5′-GAT GGA GTT CAT AAC CCA TCC AAA G-3′ [strain 1170-1146 MN]. The final PCR product consisted of a fragment of 750 bp.

Solid-phase DNA sequencing was performed according to Wahlberg and collaborators (25) by using primers JA100 and biotinylated RT 136 for final amplification of the PCR products. The fluoresceinated sequencing primers were RIT 136 (5′-FITC-GAT GGA GTT CAT AAC CCA TCC AAA G-3′) and RIT 227 (5′-FITC-AAT CTG TTG ACT CAG ATT GG-3′). The sequence reactions of both immobilized and supernatant strands were derived by using T7 DNA polymerase. Sequences were determined with an automated laser fluorescent sequencing apparatus (Pharmacia, Uppsala, Sweden). Sequences were analyzed by using Genetics Computer Group (University of Wisconsin) software (8). Cluster analysis was performed by analyzing pairwise aligned sequences. Gaps were excluded from the calculations.

RESULTS

IC_{50}s and their correlation to the presence of mutations in blood and CSF isolates. All blood and CSF isolates obtained prior to AZT therapy were sensitive to the drug (IC_{50}, <0.3 μmol/liter). Four of the six CSF isolates obtained during treatment (10 to 38 months) were highly resistant (IC_{50}, >1.85 μmol/liter) and two were partially resistant (0.4 μmol/liter). Similarly, four of the paired isolates from blood were highly resistant (2.5, 6.8, 8.7, and 11 μmol/liter) and two were partially resistant (0.38 and 0.3 μmol/liter) to AZT.

The patterns of mutations in blood and CSF are shown in Table 2. Five of the six patients had mutations known to confer AZT resistance: in four patients the mutations were found in both blood and CSF, and in the remaining patient the mutation was seen only in the blood sample. HIV-1 isolates from the blood and CSF of one patient (patient 3) showed the same combination of the two mutations at the levels of codons 70 and 215 in blood and CSF, whereas the isolates obtained from the remaining four patients presented a different pattern of mutations in the two compartments. Blood and CSF isolates obtained before AZT treatment were also sequenced. All of these isolates were shown to be of the wild type except the isolate obtained at time zero from patient 4 (codon 41). The sequence of this isolate was repeated on several occasions. It should be mentioned in this context that HIV-1 isolates carrying one of the codons linked to AZT resistance have been previously isolated from untreated patients (21). A correlation between the sensitivity to the drug in vitro and the presence of amino acid substitutions in the RT gene was demonstrated for five of the six isolates from the CSF. In the isolate obtained from the CSF of patient 5, the high IC_{50} did not correlate with the presence of mutations in the RT gene.

The IC_{50} increased from 0.015 to 6 μmol/liter in the CSF isolates 18 months after the beginning of therapy, whereas the corresponding values for the isolates from blood were 0.065 and 0.3 μmol/liter. The last isolate from the CSF was, there-

TABLE 2. RT mutations in blood and CSF isolates from AZT-treated patients

Mutation	RT codon	Patient 1 (13 mo)[a]		Patient 2 (29 mo)		Patient 3 (38 mo)		Patient 4 (10 mo)		Patient 5 (18 mo)		Patient 6 (24 mo)	
		Blood	CSF	Blood	CSF	Blood	CSF	Blood	CSF	Blood	CSF	Blood	CSF
M→L	41	+	+	−	−	−	−	+	−	−	−	−	+
D→N	67	−	−	−	+	−	−	−	−	−	−	−	+
K→R	70	−	−	+	+	+	+	−	−	−	−	+	+
T→F/Y	215	+	+	+	+	+	+	−	−	−	−	−	+
K→Q	219	−	+	+	+	−	−	−	−	−	−	−	−

[a] Months of therapy.

TABLE 3. Amino acid variations in blood and CSF before and during AZT treatment

Patient	% Variation (mo between samples)			
	CSF[a]→CSF[b]	Blood[a]→blood[b]	Blood[a]→CSF[a]	Blood[b]→CSF[b]
1	2.7 (13)	4.2	4.2	2.2
2	1.8 (29)	3.0	1.8	1.4
3	4.7 (38)	4.1	3.7	6.4
6	4.1 (24)	4.1	3.7	6.4
Avg	3.3	3.8	3.3	4.1

[a] Before therapy.
[b] During therapy.

fore, considered highly resistant, in spite of the lack of mutated codons in the RT gene. The IC_{50} data for all remaining isolates from blood correlated with the presence or absence of mutations (Tables 1 and 2).

Appearance of mutations in relation to time. We also studied the appearance of the RT mutations in relation to the duration of AZT therapy. Early during treatment, a virus prevalently mutated at the level of codons 41 and 215 appears to be present in both CSF and blood. After 20 months of therapy, the amino acids more prone to mutate became codons 70 and 215. Interestingly, the codon 67 mutation was present only in two of the isolates obtained from the CSF of two patients. The corresponding isolates from blood did not show this substitution.

Rate of RT mutations in blood and CSF over time. In order to study the rate of RT mutation intrapatient over time, we calculated the percentage of amino acid variations in the sequences of HIV-1 isolates from the same patient in separate compartments before and during AZT treatment (Table 3). The mutations involved in the AZT resistance phenotype were not included in the analysis. The results obtained with isolates from four patients were evaluated. Virus isolates from patient 5 were not included because there was no variation observed over time in the different compartments. Patient 4 was not included because the CSF isolate pretreatment was not available. As shown in Table 3, the percentages of amino acid variations were approximately equal when the same compartment, e.g., CSF versus CSF, was analyzed over time. After 1 to 3 years of treatment, two of the four patients showed an increased rate of amino acid substitutions between the two distinct compartments (patients 3 and 6), whereas two patients showed a reduction. The mean variation in the distinct compartments, over time or between compartments, was approximately the same (3.3 to 4.1%). Interpatient variations ranged from 2.2 to 5.3% when isolates from the blood were compared prior to AZT treatment.

DISCUSSION

The invasion of the brain by HIV-1 occurs early and regularly after infection (7). Any therapeutical approach aimed to control HIV-1 replication will have to take into consideration the existence of barriers that restrain the entry of drugs into the brain. That AZT crosses the blood-brain barrier has been documented in a previous study (4). In fact, 4 h after AZT administration, the drug concentration in the CSF was half of that present in plasma (11). The focus of our study has been to assess whether AZT resistance occurs in HIV-1 strains present in the brain or if a reservoir for wild-type virus is present in this organ. In order to compare the degree of AZT sensitivity of the isolates with the presence of mutations, we have used cultured material. Our study may therefore be biased by the effect of coculture on the selection of variants from the quasispecies present in the patient.

Isolates from the CSF were shown to be resistant to AZT in vitro. The analysis of the RT sequences at different times during therapy has shown that the amino acid substitutions involved in AZT resistance in the brain are the same as those documented for resistant viruses from the blood (20). High levels of resistance 2 years after initiation of treatment were associated with the presence of mutations at codons 70 and 215.

Interestingly, the isolate obtained from the CSF of patient 5, 18 months from the beginning of therapy, displayed a high degree of resistance to the drug in vitro, but no mutations could be observed in the RT sequence. This change in susceptibility to AZT is not easy to explain. The analysis of the RT sequences of the CSF isolates obtained before and after AZT treatment revealed no differences. Similarly, we could not detect any switch in the biological phenotype of the viruses during this period (data not shown). The assay used to evaluate the biological phenotype was growth and syncytium formation in MT-2 cells (13). It is possible that other mutations located outside the region we sequenced, and occurring less frequently than the five AZT positions described so far, may confer a resistance phenotype in vitro (24).

In four of the six patients studied, the patterns of mutations in the CSF isolates were different from those found in the blood. This indicates that the virus populations in the two compartments are different and may have evolved apart from each other. These results are in accordance with previous observations obtained after examining V3 loop sequences from patients at different clinical stages of the disease (10). In the latter study we could not identify amino acid positions representative of tissue source. The percentage of V3 amino acid variations between blood and CSF isolates, however, increased according to the clinical stage, suggesting adaptation of the virus in the nervous system. The number of changes in the RT sequences did not appear to increase with time and was not cumulative. In fact, whenever the isolates present in blood and CSF compartments were compared over time, the mean variation was similar to what we found to represent interpatient variation or what has been estimated to represent RT infidelity (16). This observation reinforces the hypothesis that the intrapatient variation noticed at the level of the envelope protein gp120 may, at least in part, be due to immunomodulation.

In conclusion, our data show that HIV-1-resistant strains can be found in the brain compartment of patients undergoing AZT therapy. We used CSF isolates in order to compare amino acid sequences before and during treatment. However, differences in the RT genotypes present in CSF and brain parenchyma may exist, as indicated by the distinct biological properties of isolates obtained from brain and CSF (14). The mutations reported to be involved in the development of AZT resistance in the blood compartment can be found also in the brain isolates. The appearance of these mutations is a distinct event in these two compartments; thereafter, a convergence into viruses mutated at codons 70 and 215 can be noticed in both blood and brain.

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