Failure of Stavudine-Lamivudine Combination Therapy in Antiretroviral-Naive Patients With AZT-Like HIV-1 Resistance Mutations

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To analyze the clinical relevance of AZT resistance mutations in AZT-naive patients, 56 HIV-1 seropositive patients treated for 18 months with stavudine/lamivudine (27 patients) or AZT/lamivudine (29 patients) were studied. AZT-like resistance mutations were found in 13 out of 29 (44%) patients treated with AZT/lamivudine and in 11 out of 27 (40%) patients treated with stavudine/lamivudine. No stavudine or multi-drug resistance mutations were detected. After 26 months of treatment more than 60% of patients showed a virological failure. Among 10 patients failing treatment with stavudine/lamivudine, 9 had AZT-like resistance mutations. The phenotypic test, performed on HIV-1 strains isolated from six of these nine patients, showed a resistance to AZT in five isolates and to stavudine in two isolates. The genotypic pattern of the latter two isolates showed the combined mutations M184V plus R211K and L214F. AZT-like resistance mutations in AZT-naive patients seem to correlate with a virological failure during long-term stavudine therapy. *J. Med. Virol. 65:631–636, 2001.*   © 2001 Wiley-Liss, Inc.

KEY WORDS: AZT resistance; antiretroviral therapy; stavudine-lamivudine combination therapy

INTRODUCTION

Current treatment guidelines for human immunodeficiency virus type-1 (HIV-1) infected individuals recommend a combination of two nucleoside reverse transcriptase inhibitors and a protease inhibitor or non-nucleoside reverse transcriptase inhibitor [Carpenter et al., 2000]. These combinations of antiretroviral drugs have been shown to decrease plasma HIV RNA level below the quantification limit of current viral load assays in the majority of patients, thus limiting the development of resistance and providing a long-lasting clinical benefit [Hammer et al., 1997; Hogg et al., 1997; Hogg et al., 1998].

Double nucleoside therapy is obsolete because only a small number of patients will profit from it in the long-term and it is not possible to predict in advance which kind of patients can be treated adequately with only two nucleoside reverse transcriptase inhibitors. Nevertheless, in the clinical practice, many physicians have experience with patients who underwent long-term treatment with a dual combination therapy with clinical and immunological benefits.

Previous studies [Foudraine et al., 1998; Molina et al., 1999] demonstrated the efficacy of lamivudine in dual nucleoside combinations for the treatment of HIV-1 infection in antiretroviral-naive patients. Particularly, these studies showed comparable virological effects of lamivudine associated with stavudine or zidovudine (AZT). The virological response to stavudine, however, is attenuated among people with prior exposure to AZT

[Katlama et al., 1998] depending either on the presence of AZT-resistance mutations [Izopet et al., 1999] or on pharmacologic antagonism [Sommadossi et al., 1998]. Moreover, a significant minority of AZT-naive patients receiving long-term stavudine and didanosine eventually develops mutations at codons conferring AZT resistance [Ross et al., 1999; Coakley et al., 2000]. The prevalence of these AZT-like mutations in stavudine-treated patients remains uncommon and it probably only occurs after long-term virological failure.

The aim of the study was to examine the prevalence and the clinical relevance of AZT- resistant mutations in AZT-naive patients treated with lamivudine plus stavudine that are experiencing clinical and virological improvement.

MATERIALS AND METHODS

Patients

Twenty-seven HIV-1 seropositive patients, receiving a combination of stavudine 40 mg (or 30 mg for patients weighing < 60 kg) plus lamivudine 150 mg twice daily (stavudine/lamivudine group) and 29 patients treated with AZT 200 mg three times daily plus lamivudine 150 mg twice daily (AZT/lamivudine group), were enrolled in the study. The inclusion criteria were a progressive decline of plasma HIV-1 RNA to $< 10,000$ copies/ml and an increase of the CD4 + cell count (> 50 cells/ml from base line value) after at least 6 months of treatment. Patients who had received any antiretroviral treatment before the combination of two analogue nucleosides or had malignancies that required cytotoxic chemotherapy were excluded from the study.

Subsequent monthly medical examinations were scheduled after the start of therapy. At each time a complete physical examination was carried out and blood was taken for immunological and virological assays and for routine safety laboratory parameters.

Informed consent was obtained from all patients participating in this study.

Laboratory Monitoring

A blood sample was obtained from patients at enrollment.

HIV-RNA was quantified with the Amplicor Monitor Assay (Roche Molecular System Branchburg, NJ). When the level of plasma HIV-RNA dropped below 400 copies/ml, separate aliquots of plasma were assayed using the Ultradirect Assay (limit of detection: 20 copies/ml).

HIV was isolated from CD8 + depleted peripheral blood mononuclear cells (PBMCs). Briefly, negative selection with magnetic beads (Miltenyi Biotec GmbH, Germany) was used to remove CD8 + T-cells from PBMCs and the negative fraction, activated in the presence of 100 U/ml of human recombinant interleukin-2 and 5 μg/ml of phytohemagglutinin (PHA, Sigma, St. Louis, MO), was cocultured with 10^7 CD8 + depleted PBMCs combined from two seronegative

donors. The cultures were placed in a humidified chamber at $37°C$ with 5% CO_2 and maintained for 60 days and monitored twice a week for p24 antigen production using a commercially available enzyme immunoassay (Abbott Laboratories, North Chicago, IL). A culture was considered positive if the concentration of p24 exceeded 1,000 pg/ml in two consecutive determinations.

Positive supernatants were harvested by centrifugation and stored in liquid nitrogen.

Viral isolates were tested for sensitivity to analogue nucleoside reverse transcriptase inhibitors. Briefly, PHA-stimulated donor PBMCs (4×10^6 cells) were infected with 2 ml of medium containing a viral stock adjusted to a multiplicity of infection of 2,000 $TCID_{50}/$ml. After a 2-hr adsorption period, aliquots of the cells washed twice in PBS were put into a 96-well plate containing five different concentrations of AZT (0.001, 0.01, 0.1, 1 and 5 μM) or stavudine (0.005, 0.05, 0.5, 5, and 25 μM). All culture assays were carried out in quadruplicate and monitored for p24 antigen production for 7 days after infection. The 50% inhibitory concentrations (IC_{50}) of antiviral drug were determined based on comparative growth of isolates in untreated control cultures. For phenotypic drug susceptibility testing, the strains HIV-1$_{MP27}$ and HIV-1$_{GA61}$, isolated from antiretroviral naive patients in 1986, served as the susceptible control. HIV-1$_{ST543}$ isolate with the complete Q151M multinucleoside resistance complex (A62V, V75I, F77L, F116Y, and Q151M) served as the resistant control.

HIV isolates were considered resistant to drugs for $IC_{50} > 10$-fold compared with the IC_{50} of drug-sensitive strains and, in all cases, resistant to AZT for $IC_{50} > 0.1$ μM and to stavudine for $IC_{50} > 0.5$ μM.

Polymerase Chain Reaction and Direct Sequencing of Reverse Transcriptase

Three million of PBMCs from the patients were suspended and lysed in 400 ml of lysis buffer containing 10 mM Tris-Hcl [pH 8.3], 1 mM EDTA, 0.5% Triton X-100, 0.001% SDS, 300 mg/ml proteinase K. Lysed cells were digested with proteinase K overnight at $37°C$ and at the end point the enzyme was inactivated for 15 min at $94°C$. The lysates were stored at $-20°C$ until they were used. In the first amplification for reverse transcriptase gene analysis a fragment of 930 bp was obtained with the following oligonucleotide, JA99 5'-GGG GGA ATT GGA GGT TTT ATC AAA G-3' and MM4 5'-TTC TGT ATG TCA TTG ACA GTC CAG C-3' (0.2 mM), and 0.2 mM dNTPs. Polymerase chain reaction was performed in 40 cycles, each consisting of denaturation at $94°C$ for 30 sec, annealing at $60°C$ for 30 sec, and elongation at $74°C$ for 30 sec, plus a final extension at $74°C$ for 10 min. Five microliters were used as template for the second amplification performed in the same condition by additional 30 cycles using the inner primers JA100 5'-GAC CTA CAC CTG TCA ACA TAA TTG G-3' and MM3 5'-GAT GGA GTT CAT AAC

CCA TCC AAA G-3′. The final product consisted of a fragment of 750 bp [Izopet et al., 1999].

For sequencing, 200 ng of the polymerase chain reaction product and a cycle sequencing kit (Prism Ready Reaction Big Dye Terminator; Applied Biosystems, Foster, City, CA) with DNA polymerase were used. The conditions for 25 cycles were 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. To obtain forward and reverse sequences of the polymerase chain reaction products, sequencing primers used in separate reactions were JA100 and MM3. Nucleotide sequencing was performed for codons 1–240 of the HIV-1 reverse transcriptase gene. Detection of sequencing products and generation of sequence data was done on ABI 310 automated sequencer (Applied Biosystems)

Gene sequences were analyzed with DNAsis software and were related to the HIV-1$_{LAI}$ sequence (GenBank accession number K02013). Details of key mutations in the pol gene associated with reduced sensitivity to antiretroviral treatment were obtained from literature.

Statistical Analysis

Statistical analysis of the correlation coefficients was carried out using the Fisher's exact test (P two-tailed); the Student's t test was used for continuous measurements to test relationships in paired analysis. Group means were compared using analysis of variance.

RESULTS

No significant differences in demographic (age, gender, or HIV transmission risk group), virological and immunological parameters between patients treated with AZT/lamivudine and stavudine/lamivudine were found. At base line, however, patients treated with stavudine/lamivudine had a lower CD4 + cell count and a higher viral load (359 ± 161 CD4 + cells/μl and 4.60 ± 4.85 log HIV-RNA copies/ml) than patients treated with AZT/lamivudine (472 ± 318 CD4 + cells/μl and 4.48 ± 4.7 log HIV-RNA copies/ml).

After 18 months of therapy no difference in terms of immunologic and virological parameters was detected between the two groups of patients (Table I). Particularly, 12 patients, six in each group, had less than 400 HIV-RNA copies/ml, three of them with undetectable levels (< 20 copies/ml).

Mutations in the HIV-1 RT region associated with drug resistance were detected in PBMC of 31 out of 56 (55%) patients. No significant difference in the presence of resistance mutations was found in the two treatment arms even if AZT-treated patients had a higher prevalence of analogue nucleoside resistance mutations. Particularly, mutations at codons conferring AZT resistance were found in 13 out of 29 (44%) patients treated with AZT/lamivudine and in 11 of 27 (40%) patients treated with stavudine/lamivudine. The AZT-resistant mutations M41L and K70R were the detected most frequently in stavudine/lamivudine and AZT/lamivudine groups, respectively.

Although a higher prevalence of the M184V mutation, related to lamivudine high resistance, was observed in patients treated with AZT/lamivudine compared with patients treated with stavudine/lamivudine (41% and 14%, respectively), this difference approached a weak statistical significance ($P = 0.05$). Only one patient in the AZT arm showed the dual combined mutation M184V plus R211K that confers high-level resistance to both AZT and lamivudine. Mutations L100I and Y181C, conferring resistance to non-nucleoside reverse transcriptase inhibitors, were observed in two patients treated with stavudine/lamivudine and AZT/lamivudine, respectively.

No mutation related to stavudine resistance was observed in patients treated with a stavudine-including regimen; on the other hand, the I178M mutation associated with stavudine treatment failure [Danehower et al., 1998; Lin et al., 1999] was detected in one patient in the AZT arm.

The sequencing of the reverse transcriptase-gene was undertaken in plasma samples of 11 individuals with a viral load of more than 2,000 HIV-RNA copies/ml. Drug resistant mutations were detected in all samples and a substantial correlation between HIV-1 drug-associated resistance mutations in plasma and in PBMCs was found. Particularly, a difference between HIV-1 drug resistance mutations detected in plasma and in PBMCs for resistant associated codons was found in only two patients (M184V and T215Y respectively).

Table II shows immunological and virological parameters of patients after an average period of 18 months of therapy according to the presence of mutations conferring resistance to nucleoside reverse transcriptase inhibitors. No significant difference in mean CD4 cell count and viral load was found between patients with a strain resistant or sensitive to drugs. Patients

TABLE I. Immunological and Virological Parameters of Patients After an Average Period of 18 Months of Therapy According to Treatment Arm

	AZT/lamivudine treatment (N = 29)	Stavudine/lamivudine treatment (N = 27)
Months of treatment[a]	18 ± 5.8	18 ± 13
CD4 + cell count ($\times 10^6$/l)[a]	598 ± 300	511 ± 230
HIV-RNA (log$_{10}$ copies/ml)[a]	3.58 ± 3.67	3.57 ± 3.42
Patients with < 400 HIV-RNA cp/ml (%)	6 (20)	6 (22)
Patients with < 20 HIV-RNA cp/ml (%)	1 (3)	2 (7)

[a]Values are mean ± standard deviation.

TABLE II. Immunological and Virological Parameters of Patients After 18 Months of Therapy According to Treatment and Genotypic Resistance to Analogue Nucleosides

	AZT/lamivudine treatment		Stavudine/lamivudine treatment	
	Sensitive (N = 11)	Resistant (N = 18)	Sensitive (N = 14)	Resistant (N = 13)
Months of treatment[a]	17 ± 6	19 ± 5	16 ± 7	18 ± 12
CD4 + cell count $(\times 10^6/l)$[a]	553 ± 262	625 ± 325	516 ± 256	477 ± 223
HIV-RNA $(\log_{10}$ copies/ml)[a]	3.28 ± 3.48	3.69 ± 3.61	3.18 ± 3.28	3.55 ± 3.48
HIV-RNA decrease from base line $(\log_{10}$ copies/ml)	-1.44	-0.88	-1.48	-1.02
Patients with < 400 HIV-RNA cp/ml (%)	4 (36)	2 (11)	5 (35)	1 (7)
Patients with < 20 HIV-RNA cp/ml (%)	0	1 (5)	2 (14)	0

[a]Values are mean ± standard deviation

with resistant mutations, however, had a lower decrease in viral load with respect to the base line value and a longer treatment period than patients with the wild-type virus. Moreover, nine out of 25 (36%) patients with the wild-type virus reached an HIV-RNA level < 400 copies/ml compared with three out of 31 (9%) patients with resistant mutations ($P = 0.03$). Only three patients had an undetectable viral load (< 20 copies HIV-RNA/ml): two patients infected with the wild-type virus treated with stavudine/lamivudine, and one patient with the AZT resistance mutation K70R treated with AZT/lamivudine.

The characteristics of 47 individuals with an average period of 26 months of follow-up are showed in Table III. At the end of the study, a treatment failure, defined as viral load equal or greater than the base line value, was detected in the majority of patients: 17 (65%) individuals in AZT arm and 13 (62%) individuals in stavudine arm. A wild-type virus was detected in 13 out of 17 (76%) patients responding to treatment compared with six out of 30 (20%) patients failing therapy. Among 10 patients with nucleoside reverse transcriptase inhibitors resistance mutations and failing treatment with stavudine/lamivudine, nine had AZT-like resistance mutations alone or in combination with M184V mutation (3 patients). One patient with

M41L mutation showed a persistent low level of viral load (320 HIV-RNA copies/ml) during stavudine treatment.

The isolation of HIV-1 strain yielding a viral titre of more than the prerequisite 4,000 $TICD_{50}$ to perform phenotypic assay, was obtained in 17 patients. Particularly, in six patients failing treatment with stavudine/lamivudine and with AZT-like resistance mutations alone, the phenotypic assay showed a resistance to AZT in five individuals and a resistance to stavudine (IC_{50} 19.2 and 22.3 µM) in two patients. Different from the archival genotype of the PBMCs of patients, the genotypic pattern of the two stavudine resistant isolates showed the combined mutations M184V plus R211K and L214F.

DISCUSSION

The aim of the study was to evaluate the prevalence and the clinical relevance of AZT-like resistance mutations in AZT-naive patients receiving stavudine plus lamivudine. After 26 months of treatment the majority of patients (62%) presented a treatment failure showing viral load levels equal to the base line value or higher. One or more mutations conferring AZT resistance were developed in 40% of patients treated with

TABLE III. Immunological and Virological Parameters in 47 Patients After 26 Months of Therapy According to the Outcome

	AZT/lamivudine treatment		Stavudine/lamivudine treatment	
	Efficacious (N = 9)	Failing (N = 17)	Efficacious (N = 8)	Failing (N = 13)
Months of treatment[a]	25 ± 7	27 ± 5	24 ± 7	27 ± 10
CD4 + cell count $(\times 10^6/l)$[a]	720 ± 256	487 ± 198	489 ± 236	513 ± 208
HIV-RNA $(\log_{10}$ copies/ml)[a]	2.58 ± 2.57	4.16 ± 4.21	2.72 ± 2.7	4.22 ± 4.36
Patients with < 400 HIV-RNA cp/ml (%)	6 (66)	0	4 (50)	0
Patients with RT genotype				
Wild type	6	3	7	3
Resistance mutations	3	14	1	10
AZT resistance mutations alone	1	5	1	6
M184V alone	0	4	0	1
AZT resistance mutations + M184V	1	6	0	3
Other	1[b]	0	0	0

[a]Values are mean ± standard deviation.
[b]I178M.

stavudine/lamivudine and all but one patient with AZT-like mutations showed a virological failure.

In AZT-naive patients treated with stavudine a reduction of AZT- [Lin et al., 1994; Pellegrin et al., 1999] and stavudine-susceptibility [Bloor et al., 1998] has been reported. Moreover, the HIV-1 strains isolated from these patients harbored AZT-like resistance mutations [Bloor et al., 1998].

In this study, two out of six HIV-1 strains isolated from patients with AZT resistance mutations alone had a reduced sensitivity to stavudine in vitro. Interestingly, in both isolates the genotypic analysis detected a combination of multiple resistant mutations (M184V plus R211K and L214F) conferring high-level resistance to both AZT and lamivudine [Kemp and Bloor, 1997] but still not related to stavudine resistance. Therefore, in comparison with wild-type virus, HIV-1 variants with AZT-resistance mutations have probably a higher replicative competence in the presence of stavudine.

Resistance mutations reported as stavudine-specific, in agreement with data reported previously [Izopet et al., 1999; Pellegrin et al., 1999], were not detected in the stavudine/lamivudine group. In one patient treated with AZT/lamivudine the I178M mutation was detected. This mutation, related to stavudine treatment failure, has been also considered a polymorphism found in a large percentage of stavudine-naive patients [Schinazi et al., 1999]. Differently from others studies [Izopet et al., 1999; Pellegrin et al., 1999], no multidrug-resistance mutations were detected: a possible explanation could be that the patients in the current study were responding to therapy in terms of low viraemia levels.

Reverse transcriptase sequence analysis was possible from plasma of only 11 individuals in this study. The low HIV-RNA level of patients could explain the low rate of reverse transcriptase plasma sequencing. Nevertheless, similarly to other study [Devereux et al., 2000], a substantial correlation between plasma and PBMCs resistance mutations was detected.

In this study, stavudine-lamivudine association was at least as effective as AZT-lamivudine combination, although both treatments could be considered suboptimal in term of suppression of HIV-1 replication and in CD4 + cell recovery. A limited number of patients in both groups reduced HIV-RNA levels under the limit of detection of the assay and 55% of patients developed antiretroviral drug resistance. Nevertheless, some patients had a very low viral load despite the emergence of nucleoside reverse transcriptase inhibitors resistance. This observation could be explained by a decreased viral fitness of the HIV-1 variants harboring drug resistance mutations [Harrigan et al., 1998]. Moreover, the genotypic analysis was carried out on PBMCs whereas viral RNA was measured in the plasma compartment reflecting the possible difference between cell-associated provirus, representative of the archival genomic library, and actively replicating virus. Considering the limits of the genotyping test, however, further investigations based on phenotypic

analysis are needed to determine the susceptibility profile of the HIV-1 mutants selected by combination therapies.

Unlike other studies [Foudraine et al., 1998; Ross et al., 1999], only few M184V mutations with a significantly higher prevalence in AZT/lamivudine group were found. A possible explanation can lie in the selection of patients with very low levels of viral load over 18 months of therapy with a hypothetical protective role of stavudine on the emergence of lamivudine resistance. In fact, the M184V mutation is able to escape reverse transcriptase-inhibition more efficiently during AZT/lamivudine than stavudine/lamivudine treatment [De Wolf et al., 2000].

The resistance pattern of HIV-1 strains was not analyzed before the start of therapy and we cannot affirm if the AZT-like mutations were already present before treatment or were selected during the therapy. Nevertheless, a relevant number of patients failing stavudine/lamivudine therapy harbored an HIV strain with AZT-like resistant mutations. This phenomenon, occurring in 40% of patients seems to correlate with a virological failure during long-term stavudine treatment.

In the absence of genotypic and phenotypic susceptibility testing, it may be inappropriate to recommend AZT in new antiretroviral regimens after stavudine therapy failure. The emergence of mutations related to nucleoside reverse transcriptase inhibitors is a consequence of suboptimal treatment efficacy.

ACKNOWLEDGMENTS

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