Trends in Drug Resistance Mutations in Antiretroviral-Naïve Intravenous Drug Users of Rio de Janeiro

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DNA sequencing of a *pol* gene fragment from drug-naive injecting drug users samples obtained at two time points of the Brazilian AIDS epidemic (Pre-HAART era: 1994 to early 1997, n = 27; post-HAART era: 1999–2001, n = 38) was undertaken to assess HIV-1 antiretroviral drug resistance mutations and subtyping profiles. Genotypic analysis revealed the presence of PR primary L90M, D30N, M46I, and V82A mutations in 7.9% of the post-HAART group, and a high frequency of secondary mutations (84.2%). Nucleoside RT-associated mutations were observed in 13.2%. In the pre-HAART group, a higher frequency of RT mutations was observed (22.2%) and no PR primary mutations were found, in agreement with the introduction of protease inhibitors (PIs) in therapy during the same period. The identification of 7.9% of drug-naive injecting drug users already bearing RT/PR primary resistance mutations in the post-HAART era group constitutes a major concern in terms of dissemination of drug resistant viruses. The resistance mutations profile of the individuals may reflect the context of antiretroviral treatment in Brazil at the sample collection periods (1994–1997 and 1999–2001). In spite of the differences observed in the drug resistance profiles, similar frequencies of subtype B (63.0 vs. 73.7%), F (22.2 vs. 10.5%), and recombinant B/F (14.8 vs. 15.8%) viruses were found, respectively, in the pre- and post-HAART groups. *J. Med. Virol. 78:764–769, 2006.* © 2006 Wiley-Liss, Inc.

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INTRODUCTION

HIV-1 is currently classified in, at least, 9 subtypes, 16 circulating recombinant forms (CRFs) and a large spectrum of unique recombinant genomes [Robertson et al., 2000]. Molecular epidemiological studies conducted in Brazil have shown the predominance of subtypes B, C, F, A, D, and B/F and B/C recombinant forms [reviewed in Morgado et al., 2002].

Combinations of three or more drugs from two drug classes (highly active antiretroviral therapy, HAART) can lead to prolonged virus suppression and immunologic reconstitution in individuals infected by drug-susceptible HIV-1 strains [Shafer, 2003]. Partial replication suppression periods could favor the development of drug resistance, leading to an increase of multi-drug resistant viruses transmission frequency and a high prevalence of resistant variants in newly infected individuals [Cohen and Fauci, 1998]. Suboptimal adherence to treatment could promote the selection of drug resistant viral strains [Bangsberg et al., 1997], making the resistance surveillance pivotal in populations where suboptimal adherence has been described, such as injecting drug users and other marginalized populations [Mitty et al., 2002].

The shared use of injectable drugs constitutes the second most common exposure form to HIV infection in Brazil, accounting for 15.9% of the total number of AIDS cases notified in Brazil as of June 2005 [National Ministry of Health, Brazil, 2005]. The epidemic among injecting drug users has been particularly dynamic, with marked contrasts between different regions and

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localities, overtime [Hacker et al., 2006]. Due to their exposure to parenterally and sexually-transmitted infections and difficulties of complying with clinical follow-up, injecting drug users may represent a source for the dissemination of resistant viruses.

The present study was to evaluate HIV-1 antiretroviral drug resistance mutation profiles as well as genetic diversity of protease and reverse transcriptase HIV-1 *pol* gene regions among injecting drug users from Rio de Janeiro, Brazil, recruited in two distinct moments of the Brazilian AIDS epidemic (pre-HAART era: 1994–1997, and post-HAART era: 1999–2001), in the context of two cross-sectional studies conducted by our group [Guimarães et al., 2001; Teixeira et al., 2004], which may represent a unique opportunity to assess the prevalence of primary antiretroviral drug resistance in this population.

METHODS

Study Population

Drug-naive HIV-1 infected injecting drug users from Rio de Janeiro were recruited for two cross-sectional studies conducted in 1994 to February 1997 and 1999–2001, designated here as "pre-HAART" (n = 27) and "post-HAART" (n = 38) groups. These studies were conducted with the objective to assess risk behaviors and the prevalence of HIV-1 infection in this population. Briefly, for the first study (1994 to February 1997), 175 injecting drug users were recruited, with a prevalence of 26.9% [Telles et al., 1997; Guimarães et al., 2001], while in the second study (1999–2001), 608 injecting drug users were recruited, with 7.9% of HIV seropositivity [Teixeira et al., 2004].

After signing an informed consent form, the individuals were interviewed using a standard questionnaire addressing socio-demographic data, sexual and injecting risk behaviors, and information on health. The methodology and logistics of both studies are described in detail elsewhere [Guimarães et al., 2001; Teixeira et al., 2004; Hacker et al., 2005].

HIV-1 Polymerase Subtyping and Drug Resistance Evaluation

Genomic DNA was extracted from whole blood using a phenol/chlorophorm protocol [Sambrook et al., 1989] or a DNA extraction column kit (QIAamp DNA Mini Blood Kit, QIAGEN, Valencia, California). PCR conditions are described fully elsewhere [Eyer-Silva and Morgado, 2005]. Briefly, DNA samples ($\cong$1 µg) were PCR amplified by a nested protocol, using DP10 and LR54 as outer primers, and DP16 and RT12 as inner primers, generating a 1 Kb fragment [Zazzi et al., 1993; Janini et al., 1996; Caride et al., 2000], covering both the PR and RT regions. Cycling conditions were: 3 cycles 95°C 3′, 55°C 1′, 72°C 1′; 35 cycles 95°C 1′, 55°C 45′, 72°C 1′; 1 cycle 72°C 10′. The PCR fragments were sequenced in an ABI 310 or ABI 3100 automated sequencer (Applied Biosystems, Foster City, California). Together with the

PCR inner primers, another two primers, LR49 and LR51 [Zazzi et al., 1993] were used in the sequencing reactions.

For subtyping analysis, sequences were manually edited using the DNASTAR software package (version 4.00) and the derived nucleotide sequences were aligned using the Clustal X program [Thompson et al., 1997], with a HIV-1 subtype reference set from the Los Alamos database (http://hiv-web.lanl.gov). Phylogeny was carried out based on the neighbor-joining method [Saitou and Nei, 1987], using the Kimura two-parameter algorithm for the estimation of the evolutionary distances. HIV-1 recombination profile was assessed based on bootscanning analyses [Salminen et al., 1995], using the SimPlot program (version 2.5) [Ray, 1999]. Primary and secondary drug resistance mutations described for protease and reverse transcriptase genes were determined using the Stanford Sequence Database Algorithm [http://hivdb.stanford.edu/pages/asi/] [Shafer et al., 2000] and according to the recommendations of the International AIDS Society-USA Drug Resistance Mutations Group [Johnson et al., 2005].

RESULTS

The socio-demographic characteristics of the two samples are described in detail elsewhere [Guimarães et al., 2001; Teixeira et al., 2004; Hacker et al., 2005]. All injecting drug users included in the present study had their first HIV positive serology during these surveys.

Phylogenetic analyses of the *pol* gene revealed the presence of subtype B, F, and B/F recombinant infections in both pre- and post-HAART groups. In the pre-HAART group, 17 (63%) individuals were infected with subtype B, 6 (22.2%) with subtype F, and 4 (14.8%) with recombinant viruses. In the post-HAART group, 28 individuals (73.7%) were infected with subtype B, 4 (10.5%) with subtype F, and 6 (15.8%) with recombinant viruses. No specific HIV-1 clusters, characteristic of transmission networks, was found in the present study. The subsets of potentially recombinant sequences clustered in independent branches in the phylogenetic tree, precluding a clear definition of the genetic subtype. No common pattern of recombination was found among these samples in both groups of sequences (data not shown).

The genotypic analysis of the *pol* gene of pre- and post-HAART groups is summarized in Table I. In the post-HAART group, primary PR mutations were detected in three subjects (7.9%), as follows: two with L90M and one with D30N, M46I, and V82A (Table I). Only five individuals (13.2%) presented resistance mutations in the RT gene, all associated with NRTI. In two cases, the patients carried RT resistance mutations together with primary and secondary PR mutations. D67N, T69N, K70R, T215F, and K219E mutations were detected in one person, in association with primary L90M PR mutation and L10I, D60E, L63P, I93L secondary PR mutations, indicating high-level resistance to AZT, d4T, ddC, intermediate resistance to ABC, ddI, TDF,

Maia Teixeira et al.

TABLE I. Genotypic Profiles and Frequency of Resistance-Associated Mutations in Protease and Reverse Transcriptase Genes in Pre-HAART Era Group (1994–1997) and Post-HAART Era Group (1999–2001)

	Number of individuals (%)		
Genotypic profile	Pre-HAART era group (n = 27)	Post-HAART era group (n = 38)	P-value Chi-square test
Wild-type	2 (7.4)	6 (15.8)	0.453[a]
Any resistance mutation (PR and/or RT)	25 (92.6)	32 (84.2)	0.453[a]
Only PR mutations	19 (70.4)	27 (71.0)	0.828
Only RT mutations	1 (3.7)	0	—
PR and RT mutations	5 (18.5)	5 (13.2)	0.729[a]
Frequency of resistance mutations			
Primary PI			
L90M	0	2 (5.3)	—
V82A	0	1 (2.6)	—
D30N	0	1 (2.6)	—
M46I	0	1 (2.6)	—
Total	0	3 (7.9)	—
Secondary PI			
L63P/S/Q/T/G/R/V	15 (55.6)	20 (52.6)	0.984
M36I/T	10 (37.0)	13 (34.2)	0.977
V77I	3 (11.1)	10 (26.3)	0.232
I93L	5 (18.5)	8 (21.0)	0.949
D60E/N	3 (11.1)	6 (15.8)	0.724[a]
L10I/V	2 (7.4)	4 (10.5)	1.000[a]
A71V/T	1 (3.7)	2 (5.3)	1.000[a]
K20M	1 (3.7)	0	—
I54V	0	1 (2.6)	—
Total	24 (88.9)	32 (84.2)	0.724[a]
NRTI			
M41L	4 (14.8)	0	—
T215F/Y	3 (11.1)	1 (2.6)	0.299[a]
D67N	1 (3.7)	1 (2.6)	1.000[a]
T69A/D/N	1 (3.7)	2 (5.3)	1.000[a]
K70R	1 (3.7)	2 (5.3)	1.000[a]
K219E/Q/R	1 (3.7)	3 (7.9)	0.635[a]
M184V	0	1 (2.6)	—
V118I	0	1 (2.6)	—
L210M	0	1 (2.6)	—
Total	6 (22.2)	5 (13.2)	0.503[a]
NNRTI			
V179D	1 (3.7)	0	—
Total	1 (3.7)	0	—

PIs, protease inhibitors; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitors.
[a]Fisher's exact test.

nelfinavir and saquinavir, and low-level resistance to the remaining protease inhibitors (PIs). In another case, K70R, M184V, and K219E mutations were detected in association with D30N, M46I, and V82A PR primary mutations and I54V, D60N, L63P, V77I PR secondary mutations, indicating high-level resistance to 3TC and nelfinavir, intermediate resistance to ABC, AZT, ddC, ddI, amprenavir, atazanavir, indinavir, lopinavir and ritonavir, and low-level resistance to d4T, TDF, and saquinavir (according to the Stanford algorithm output). From the remaining three patients, one with two NRTI mutations (L210M and K219R) and two with only one NRTI mutation (T69A or V118I). In the three cases, the NRTI mutations were associated with PR secondary mutations.

Contrasting with these findings, no PR primary mutation was found in the pre-HAART group, and a higher frequency of RT mutations was observed (22.2%) (Table I). Almost half of the 25 patients with PR secondary mutations and/or RT resistance mutations carried only one PR secondary mutation. Of the six patients (22.2%) presenting RT resistance mutations, five carried also secondary mutations in the PR gene. A similar proportion of individuals carrying secondary PR mutations was observed in both groups recruited before and after the HAART period (88.9 vs. 84.2%). In spite of such differences, no statistical significance was found in the frequency of PR and RT resistance mutations between the pre- and post-HAART groups (Table I).

The frequencies of PR and RT resistance mutations detected in the two groups and the distribution of viral subtypes are depicted in Figure 1. In the present study, L63P and V77I mutations predominated among subtype B samples in pre-HAART and post-HAART groups. On
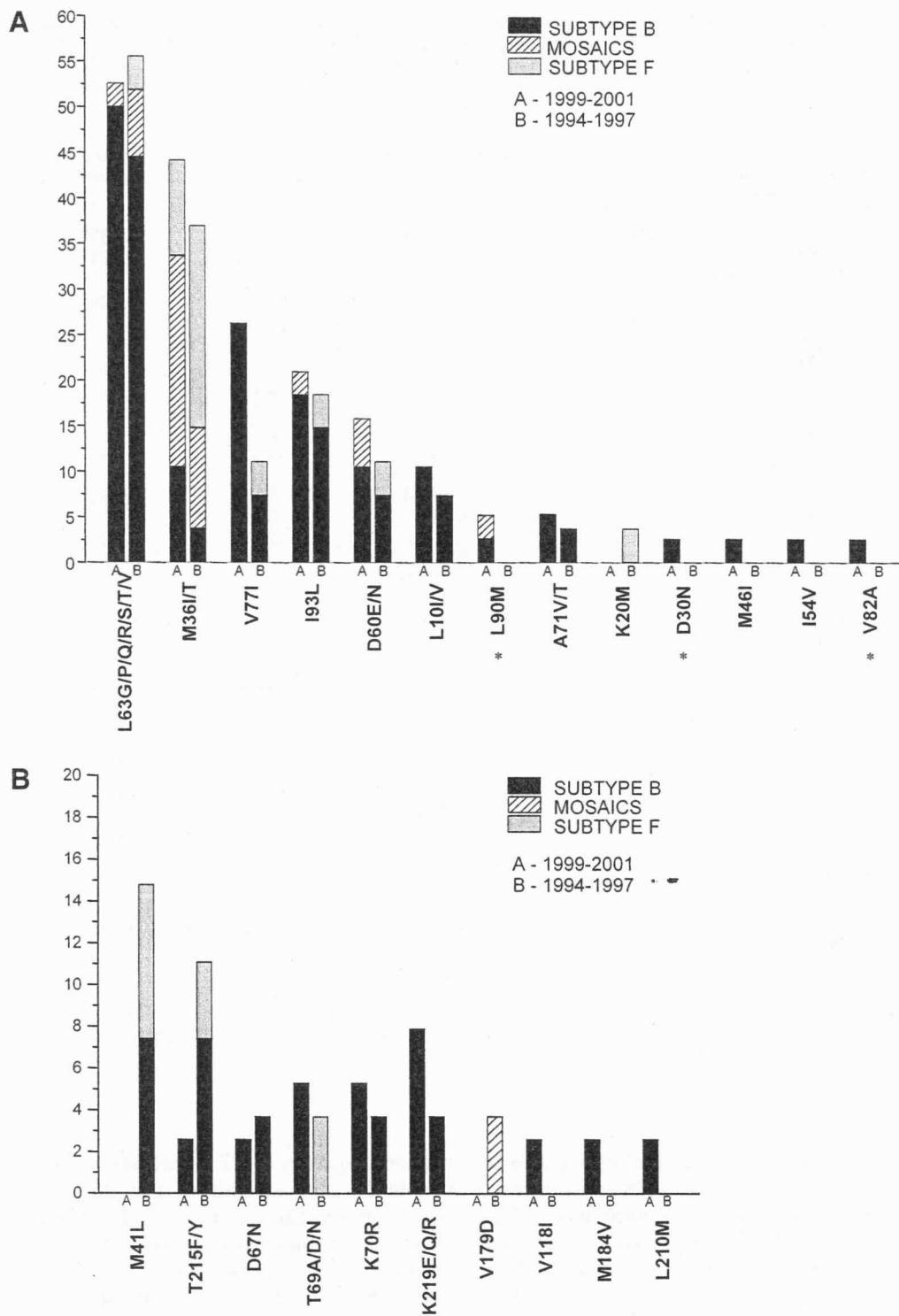
Fig. 1. Frequencies of (A) PR and (B) RT resistance-associated mutations detected in IDUs in pre- (1994 to early 1997) and post- (1999–2001) HAART eras with the distribution of viral subtypes found. * indicates D30N, V82A, and L90M primary PR mutations.

the other hand, the M36I mutation was found more frequently among subtype F and B/F mosaic genomes.

DISCUSSION

The prevalence of HIV-1 infection among injecting drug users in Brazil has been declining (29.5% in 1993–15.0% in 2005) [National Ministry of Health, Brazil, 2005], which may be related to the adoption of preventive programs directed at this population. Rio de Janeiro is one of the Brazilian States with the lowest prevalence of HIV-1 infection among injecting drug users, contributing to 3.8% of the 59.388 AIDS cases

reported for this group in the country. Following this tendency, HIV prevalences found by our surveys, carried out at two different times periods of the Brazilian AIDS epidemic, before and after the introduction of HAART document such decline [Telles et al., 1997; Guimarães et al., 2001; Teixeira et al., 2004]. The small number of samples examined in both the pre- and post-HAART eras seem to reflect the peculiar context of Rio de Janeiro, where all evidence indicates a substantial decline of HIV/AIDS in the population of injecting drug users in recent years [Bastos et al., 2005].

In the present study, the frequency of HIV-1 subtypes and the profile of antiretroviral drug mutations were studied among the HIV-1 seropositive patients diagnosed in the setting of these two surveys. The frequency of PR primary mutations (D30N, M46I, V82A, L90M) increased from zero (in pre-HAART group) to 7.9% in the post-HAART group. The identification of PR primary mutations among injecting drug users included in post-HAART group may reflect the extensive use of PIs in antiretroviral treatment in Brazil at the time of collection of the samples (1999–2001). In a context of widespread access to PI, primary PI resistance has already been described in San Francisco, USA [Hecht et al., 1998] and Geneva, Switzerland [Yerly et al., 1999] in higher frequencies than those observed in the present study. Extensive polymorphism in the protease gene was found in samples collected during both periods. Subtype-specific mutations at positions 36 and 63 have also been found as described previously [Cornelissen et al., 1997; Pieniazek et al., 2000]. On the other hand, samples collected in 1994–1997 showed a relatively high prevalence of NRTI resistance mutations (22.2%), compatible with the circulation of viral variants carrying mutations to this class of drugs (NRTIs), introduced in Brazil in the middle 1980s/early 1990s. The observed prevalence is comparable to that in Canadian injecting drug users [Salomon et al., 2000].

No PR primary mutation was found in the present study in the pre-HAART group, in agreement with the recent introduction of PIs to Brazil at that time. The findings also indicate an increase of PI genotypic resistance from the first to the second period (from 0% in 1994–1997 to 7.9% in 1999–2001), concomitant with a decrease of reverse transcriptase inhibitors (RTI) genotypic resistance (from 22.2% in 1994–1997 to 13.2% in 1999–2001). This reduction in RTI resistance prevalence may be related to a possible reduction in the fitness and transmissibility of viruses harboring these mutations overtime. The current data contrast with previous surveys conducted in Brazil among seropositive drug-naive population from voluntary counseling and testing centers [Brindeiro et al., 2003], which detected 2.24, 2.36, and 2.06% of primary mutations related to PI, NRTI, and NNRTI, respectively. Primary PI, NRTI/NNRTI mutations were not documented among blood donors from Rio de Janeiro in 1998 [Dumans et al., 2002].

Phylogenetic analyses showed that sequences are disperse, with no discernible cluster, a pattern usually observed in large transmission networks. SimPlot analyses of the putative B/F recombinant samples included in this study did not find identical recombinant breakpoints, but confirmed that all of these sequences have fragments belonging to B and F subtypes. HIV-1 B/F recombinants have been found among injecting drug users from Argentina [Espinosa et al., 2004] and other South-American populations [Thomson et al., 2000, 2002; Carr et al., 2001]. Discordant *env* and *gag* subtyping was described previously in injecting drug users from Rio de Janeiro [Teixeira et al., 2004].

The high percentage of primary mutations found in the present study may be secondary to the less than optimal management and care of local injecting drug users living with HIV/AIDS [Malta et al., 2003], coupled with risky interactions of such individuals with their drug injecting and sexual networks. Such disquieting findings constitute a major concern in terms of further dissemination of drug resistant viruses.

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