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Prevalence of Human Immunodeficiency Virus Drug Resistance Mutations and Subtypes in Drug-Naive, Infected Individuals in the Army Health Service of Rio de Janeiro, Brazil

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The prevalence of mutations that confer resistance to antiretroviral drugs was examined in 56 drug-naive, human immunodeficiency virus type 1 (HIV-1)-infected individuals from the Army Health Service in Rio de Janeiro, Brazil. No primary protease inhibitor mutations were found, but secondary mutations were observed in 51.2% of the samples. Fourteen percent of the viruses had reverse transcriptase inhibitor-associated mutations. Comparative analysis of protease secondary mutations from four different time periods in drug-naive patients in the city of Rio de Janeiro has indicated constant rates for particular mutations. Changes in CD4 cell counts and HIV viral load over time in subtype B- and non-B-infected drug-naive patients were not significantly different.

Antiretroviral drugs targeting reverse transcriptase (RT) and protease (PR) genes of human immunodeficiency virus type 1 (HIV-1) *pol* have had a positive impact on the outcomes of AIDS patients (19, 30). However, the selection of drug resistance mutations (DRM) poses one of the most serious obstacles to sustained suppression of HIV-1 (13, 25, 35, 47).

The transmission and dissemination of drug-resistant strains have major public health implications, including disrupting the efficiency of established antiretroviral treatment for HIV-1-infected patients (J. G. Garcia-Lerma, S. Nidtha, K. Blumoff, H. Weinstock, and W. Heneine, Abstr. 5th Intl. Workshop on HIV Drug Resistance and Treatment Strategies, abstr. 21, 2001; M. Gomez-Cano, A. Rubio, T. Puig, M. Perez-Olmeda, L. Ruiz, V. Soriano, J. A. Pineda, L. Zamora, N. Xaus, B. Clotet, and M. Leal, Abstr. 5th Intl. Workshop on HIV Drug Resistance and Treatment Strategies, abstr. 22, 2001; 3, 5, 9, 10, 17, 26, 27, 37). The prevalence of primary resistance mutations for any drug among recent seroconverters ranges from 0 to 17% in many industrialized countries, such as Greece, France, the United States, Italy, Canada, Germany, Spain, the United Kingdom, and Luxembourg (M. L. Chaix, M. Harzic, B. Masquelier, I. Pellegrin, L. Meyer, D. Costagliola, C. Rouzioux, and F. Brun-Vezinet, Abstr. 8th Conf. on Retroviruses and Opportunistic Infections, abstr. 755, 2001; 2, 8, 14, 16, 18, 29, 31, 40, 45, 49–51). Other studies, however, have found higher prevalences, which varied between 26 and 38% in Poland, Italy, and the United States (20, 34, 41). In Brazil, this issue has also been investigated in a few studies performed since 1996 (6, 15, 38, 42), which showed a low prevalence of mutations (0 to 5%) related to nucleoside reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs), and protease inhibitors (PIs).

The genetic variability of HIV-1 epidemiology in Brazil is complex, with subtypes B, F1, and C described as the predominant subtypes (4, 7, 11, 12, 38, 48). The role that HIV-1 subtype plays is largely unknown for non-B variants, and contradictory results have been described in different studies that attempted to show differences in progression to AIDS based on the particular infecting subtype (1, 21–24).

Fifty-six drug-naive HIV-1-positive individuals attending the Brazilian Central Army Hospital located in Rio de Janeiro, Brazil, were enrolled in the study after giving informed consent, and they were monitored from March 2000 to November 2002. The study was approved by the Brazilian Institutional Review Board (project no. 004/2001). The patients' CD4 and CD8 counts (FacsCount; Becton Dickinson, Franklin Lakes, N.J.) as well as viral loads (VLs) (NucliSens; Biomérieux, Marcy l'Etoile, France) were monitored every 3 months. Epidemiologic parameters such as gender ratios, sexual orientation, having HIV-positive partners, and Centers for Disease Control and Prevention (CDC) immunologic stage, all listed in Table 1, were compared between B and non-B groups by Fisher's exact tests. Continuous parameters (age, HIV VL, CD4 cell counts, and time from diagnosis to sampling) were compared by using Mann-Whitney U tests. Table 1 summarizes all relevant epidemiologic data. The majority of the individuals were heterosexual males. Most individuals were classified in stage A based on CDC criteria, suggesting asymptomatic infections. The duration of HIV infection in these subjects was not known, and it is likely that their infections were not recent.

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TABLE 1. Epidemiologic characteristics of drug-naive HIV-positive patients

Characteristic	Value for subtype B	Value for subtype non-B	*P* value[a]
Avg age (yr)	27.8	29.0	0.442[b]
Gender[h]			0.719[c]
Feminine	7 (18.0)	4 (23.5)	
Masculine	32 (82.0)	13 (76.5)	
Sexual orientation[h]			0.630[c,d]
Heterosexual	27 (90.0)	11 (84.6)	
Homosexual		1 (7.7)	
Bisexual	3 (10.0)	1 (7.7)	
HIV-positive partner[h]			0.336[c,e]
Yes	9 (34.6)	2 (16.7)	
No	5 (19.2)	4 (33.3)	
No response	12 (46.2)	6 (50.0)	
CDC classification[h]			0.136[c,f]
A	27 (73.0)	9 (52.9)	0.365[c,g]
B	5 (13.5)	6 (35.3)	
C	5 (13.5)	2 (11.8)	
Median VL at diagnosis (copies/ml)	34,000	32,000	0.515[b]
Median $\log_{10}$ HIV VL	4.53	4.50	
Average CD4 T-cell counts at diagnosis	459.1	439.9	0.684[b]
Infection time after diagnosis (yr)	1.14	0.95	0.322[b]

[a] *P* values were calculated for a 95% confidence interval.
[b] Mann-Whitney U test was used.
[c] Fisher's exact test was used.
[d] Shown are *P* values for heterosexuals versus those of homosexuals.
[e] Results were calculated only for "Yes" versus "No" responses.
[f] Shown are *P* values for categories A and B.
[g] Shown are *P* values for categories B and C.
[h] Values are numbers of patients. Values in parentheses are percentages.

Average CD4 counts and median $\log_{10}$ HIV VLs at diagnosis were similar in both groups (Table 1). None of the parameters analyzed could be significantly associated with a particular group. For those reasons, we assumed that both groups were comparable.

Patients' virus RNA was isolated as previously described (33). PR (whole region), RT (nucleotides 105 to 651), and gp41 immunodominant region domain were amplified through PCR. The primers and PCR conditions used were as described elsewhere (33, 43). PCR fragments were sequenced in an ABI 310 automated sequencer (Applied Biosystems, Foster City, Calif.). The determined sequences were submitted for phylogenetic analysis for HIV-1 subtype determination as described previously (7) by using ClustalW (44), PAUP version 4.0b2a (39), and SIMPLOT (36) to check for putative recombinant sequences.

The great majority of the samples had at least two virus genomic regions amplified. Forty-four out of 56 (78.6%) patients had concordant subtype assignment in the regions studied. Of those, 39 (88.6%) were subtype B isolates, 3 (6.8%) were subtype F1, 1 (2.3%) was subtype C, and 1 was CRF02_AG (2.3%) (Table 2). The remaining 12 samples revealed discordant subtypes among the regions analyzed, and they represent mosaic viruses composed of subtypes B, F1, D, and unclassified sequences.

An increase in the circulation of non-B strains in Rio de Janeiro, an area where subtype B initially prevailed, was observed when compared to results of previous studies. Dumans et al. (15) reported a prevalence of 12.2% of non-B strains in Rio de Janeiro in 1998. Brindeiro et al. (7) showed a prevalence of 19.6%. In our study, we found that 30.4% of the strains analyzed were composed of non-B subtypes. This increase was shown to be statistically significant ($P = 0.034$, chi-square test for tendency), suggesting that non-B viruses are increasing in prevalence over time in Rio de Janeiro. Moreover, the presence of subtypes never described before in Brazil, such as CRF02_AG, was observed. These results reinforce the need for HIV-1 subtype distribution surveillance policies in Brazil.

We examined the frequency of antiretroviral-associated substitutions within the RT coding region among our patients. Seven of 50 isolates (14%) had at least one NRTI-associated mutation (Table 2). The most common mutation found was M41L (4 of 50, 8%), followed by E44D (2 of 50, 4%), and V118I and T69D, both found in 2% (1 of 50) of the isolates. No NNRTI-associated mutation was observed. A number of other polymorphisms at DRM sites, not associated with drug resistance, were also observed (Table 2). Fourteen percent of the isolates analyzed harbored at least one RTI-associated mutation. When those rates were compared with those of previous DRM studies conducted in Rio de Janeiro (7, 15), we found a significant trend for increase of RT DRM ($P = 0.01$, chi-square test for linear trend). Our data suggest a continuous increase in the prevalence of RT DRM over time in the city of Rio de Janeiro. The nature of mutations found by Brindeiro et al. (7) was different from those found in our study, and it is noteworthy that NRTI-associated DRM patterns seem to have both changed qualitatively and increased in quantity over time in Rio de Janeiro.

We analyzed 21 amino acid sites in the PR region that are associated with resistance of HIV-1 subtype B to PIs approved by the U.S. Food and Drug Administration. No primary resistance mutations were found in any PR sequences. However, secondary mutations were found in 42 of 49 (85.7%) sequences at six positions. L63P was the most common secondary substitution (34.7%, 17 of 49), followed by M36I (24.5%, 12 of 49), V77I (20.4%, 10 of 49), L10I/V/F/R (10.2%, 5 of 49), K20R (6.1%, 3 of 49), and A71V/T (2.0%, 1 of 49). Mutations M36I and L10I/V/F/R were more prevalent in non-B isolates ($P < 0.001$; Fisher's exact test). Similarly to RT, a number of mutations at resistance sites not associated with resistance were found (Table 2). Since a number of PI-associated secondary mutations represent polymorphisms in non-B subtypes (46), comparison of their occurrences among different HIV-1 subtypes is becoming less important. However, we were able to search for changes over time in PI-associated secondary mutations among subtype B infections in Rio de Janeiro. Differences in subtype B isolates from different time periods (1987 to 1994; 1998; 2001; and 2000 to 2002) in the city of Rio de Janeiro were assessed by Fisher's exact tests. The substitutions found in 34 PR sequences from subtype B viruses collected in this study were compared with those found in 38 sequences collected from drug-naive individuals from 1987 to 1992 (32, 33, 43), in 41 sequences from 1998 (15), and in 41 sequences from 2001 (7). We first compared the incidence of mutations

TABLE 2. Molecular HIV-1 subtypes and genotypes of viral isolates from this study[a]

Samples	Subtypes for:			Genotype(s) at resistance-related position(s) for[b]:	
	PR	RT	gp41	PR	RT
BR02RJ01	B	B	B	**63P 77I**	WT
BR02RJ02	B	B	B	63A	**41L**
BR02RJ03	B	B	B	63A	WT
BR02RJ04	B	B	B	63S	WT
BR02RJ05	B	B	B	**63P 77I**	WT
BR02RJ06	B	B	B	**63P**	WT
BR02RJ07	B	B	B	**63P**	WT
BR02RJ08	B	B	B	**10I** 63S **77I**	103E
BR02RJ09	B	B	B	**10I** 63P **71T**	WT
BR02RJ10	B	B	B	36**L** 63P	69N
BR02RJ11	B	B	B	63A	WT
BR02RJ12	B	B	B	**63P 77I**	WT
BR02RJ13	B	B	B	**63P**	WT
BR02RJ14	B	B	B	63A	**118I**
BR02RJ15	B	B	B	63Q	179D
BR02RJ16	B	B	B	63C **77I**	WT
BR02RJ17	B	B	B	WT	**44D**
BR02RJ18	B	B	B	**63P**	WT
BR02RJ19	B	B	B	**63P 77I**	WT
BR02RJ20	B	B	B	WT	**41L** 106I
BR02RJ21	B	B	B	**63P**	WT
BR02RJ22	B	B	B	WT	98S
BR02RJ23	B	B	B	**20R**	WT
BR02RJ24	B	B	B	**63P**	41L
BR02RJ25	B	B	B	**63P**	WT
BR02RJ26	B	B	B	WT	WT
BR02RJ27	B	B	B	WT	WT
BR02RJ28	NA	B	B	NA	WT
BR02RJ29	B	B	NA	**36I**	**69D**
BR02RJ30	NA	B	NA	NA	WT
BR02RJ31	NA	B	B	NA	WT
BR02RJ32	B	B	N/S	**63P** 84M	A98S
BR02RJ33	B	NA	NA	63T	NA
BR02RJ34	B	NA	B	WT	NA
BR02RJ35	B	NA	B	**36I** 63S **77I**	NA
BR02RJ36	B	NA	NA	**63P** 77I	NA
BR02RJ37	NA	B	NA	NA	WT
BR02RJ38	B	B	NA	63A	WT
BR02RJ39	NA	B	B	NA	WT
BR02RJ40	F1	F1	F1	**36I**	WT
BR02RJ41	F1	F1	NA	**10V 20R 36I**	**41L 44D** 215D
BR02RJ42	NA	NA	F1	NA	NA
BR02RJ43	CRF02_AG	CRF02_AG	CRF02_AG	**10I** 20I **36I**	WT
BR02RJ44	C	C	C	**36I 88T**	151K
BR02RJ45	F1	B/F1	B	**10I 36I** 63A	69N
BR02RJ46	F1	NA	B	**20R** 36I	NA
BR02RJ47	B	F1	F1	63A	WT
BR02RJ48	F1	F1	B	36I	WT
BR02RJ49	B	F1	B	63A	WT
BR02RJ50	U/F1	B	B/U	WT	WT
BR02RJ51	U/F1	F1/U/B	B	36I	101R
BR02RJ52	B	F1/U/B	B	77I	WT
BR02RJ53	B/U	B	B	**36I 63P**	WT
BR02RJ54	B	B	U	**63P 77I**	WT
BR02RJ55	B	B	D	63S	WT
BR02RJ56	NA	D/B	B	NA	WT

[a] U, unclassified; NA, not analyzed; WT, wild type.
[b] Codons shown in boldface type indicate mutations accepted by International AIDS Society USA consensus; the remaining are other mutations at positions associated with resistance.

between the periods of 1987 to 1994 and of 1998, times between which the use of PIs was implemented. Since PR mutation L63P represents a significant polymorphism in subtype B viruses and may have biased those comparisons, this codon was excluded from the analysis. Although the frequencies of mutations in each specific codon did not reach statistical significance (data not shown), the frequency of isolates harboring at least one PI-associated secondary resistance mutation was higher ($P = 0.002$) in the 1998 data set (51.2%) than in the data for the 1987 to 1994 collection (18.4%), a finding that was

in agreement with the start of PI-based regimens. When comparing post-PI time periods (1998, 2001, and our data set), the number of isolates with at least one mutation has shown a trend toward decreasing ($P = 0.065$, chi-square test for tendency). This decrease may be the result of more efficacious drug regimens, which prevent the generation of new mutations, or of fewer transmissions of resistant viruses. The prevalence rates for each specific secondary mutation position were in general maintained over time, suggesting a differential impact of each position on the relative viral fitness compared to that of wild-type strains. We can envisage a scenario in which there is a dynamic equilibrium between the generation and/or transmission of these mutations and their disappearance in drug-naive individuals to restore the fitness of infecting viruses.

Patients infected with B and non-B strains were monitored for up for 32 months (median follow-up time, 19.5 months) by using CD4 cell counts and VL measures as markers. T-cell-number determinations were performed in 50 μl of whole blood-EDTA in a FACSCount apparatus and kit (Becton Dickinson) according to the manufacturer's specifications. HIV VL estimations were conducted by the nucleic acid sequence-based amplification technique using the NucliSens technology (Biomérieux), also per the manufacturer's protocol. Statistical evaluations of variations of those parameters included only patients for which at least three independent and consecutive measurements were available for each parameter. For each patient analyzed, a linear-regression curve was calculated, and its slope was determined. Patients were then pooled according to their subtype assignment (B or non-B), and median slope values were calculated for each group. CD4, CD8, and VL slope averages for each group were compared by using a Mann-Whitney U test. Statistical measurements and comparisons were performed with the statistical package SPSS for Windows and with Epi-Info (CDC). There was no significant difference between initial CD4 cell counts and VL measurements at time of diagnosis ($P = 0.684$ and 0.515, respectively; Table 1). Similarly, times of follow-up in both groups (31.5 versus 29.8 months) were almost identical and were thus of no significant difference. When the rates of CD4 cell decline were deduced for both groups, no significant difference was found ($P = 0.931$). Similarly, the rate of VL increase throughout the time after diagnosis was similar in both groups ($P = 0.544$). Although our study was not able to monitor patients from the time of seroconversion, most of our subjects were classified as being in CDC stage A, i.e., asymptomatic, and we believed them to be in the first few years of HIV infection. Moreover, the average rate of $CD4^+$ cell decline for B (152 cells/year) and non-B (124 cells/year) subtypes, as well as the VL increase per year (0.146 $\log_{10}$/year in B versus 0.255 $\log_{10}$/year in the non-B group), was comparable with results of other studies done in developed countries with subtype B-infected individuals in the first few years after seroconversion (28). Although our data do not suggest differences in rates of CD4 T-cell decline and HIV VL increase over time in subjects infected with viruses of subtypes B and non-B, it is possible that our limited sample size did not have enough statistical power to reveal the presence of possible subtle differences. Discordant significances have been reported in studies by Kaleebu et al., in which statistically significant differences in CD4 T-cell decline among distinct HIV-1 subtypes were only noticed when the number of subjects analyzed increased from 100 to more than 1,000 (21, 22).

In view of the great genetic diversity observed for HIV-1, implementing a comprehensive molecular epidemiological survey system that can trace the trend of a country's epidemic over time is crucial. Differences in important biological features of distinct HIV-1 subtypes are just now being explored, and their knowledge and understanding are vital to designing better preventive and therapeutic intervention strategies.

Nucleotide sequence accession numbers. The sequences determined in the course of this work were reported to the GenBank database (accession numbers AY285014 to AY285160).

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