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HIV-1 Diversity in Brazil: Genetic, Biologic, and Immunologic Characterization of HIV-1 Strains in Three Potential HIV Vaccine Evaluation Sites

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Summary: The Brazilian Network for HIV Isolation and Characterization was established for the surveillance of HIV variability in Brazil. Here, we report characterization of HIV strains and virus-specific immune responses from 35 clinical samples collected from three potential HIV vaccine sites. Three genetic subtypes of HIV-1 were identified by heteroduplex mobility assay (HMA) B (in 82.9% of the samples), F (14.3%), and C (2.9%). Phylogenetic analysis based on the C2V3/*env* DNA sequence from all 25 specimens examined was 100% concordant with HMA results. Four variants of subtype B with different tetrapeptides at the tip of the V3 loop were found: the GPGR motif (North American), GWGR motif (Brazilian B″), and two minor variants, GFGR and GPGS, as previously detected. No significant association was found between HIV-1 subtypes and the mode of transmission or biologic properties of HIV-1 isolates (derived from 88.6% of the specimens). Only 5 of 16 isolates studied were neutralized by the autologous sera. Consistent with previous results, no relation between viral subtype and peptide enzyme-linked immunosorbent assay (ELISA) seroreactivity or neutralization was evident. This study also demonstrated the effectiveness of the collaborative approach followed by Brazilian scientists when addressing a complex subject such as HIV variability. **Key Words:** HIV-1—Polymorphism—Molecular epidemiology—Brazil.

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As of December, 1998, >47 million people have become infected with HIV (1). Brazil is one of the most affected Latin American countries with >500,000 people living with HIV/AIDS (1,2). In Brazil, only HIV-1 has been clearly detected so far (3) and evolution of the HIV

epidemic reflects global trends. Initially, HIV transmission occurred primarily among men who have sex with men (MSM); during the second phase, drug injection played an important role and, at present, an increasingly heterosexual spread of HIV-1 has been observed (4).

The HIV pandemic is heterogeneous and one of the factors that may have an impact on the epidemic's evolution is related to the extensive variability of HIV (5–8). Genetic analyses of HIV-1 have resulted in classification into at least eight genetic subtypes, designated from A to J and four circulating recombinant forms (A/E, A/G, A/I/G, and A/B) that constitute a major group (M group) (9,10). Two additional groups, highly divergent from HIV-1 M group were later identified and termed the outliers (O) (11,12) and N (non-M and non-O) groups (13). In addition, genetic recombination between different M subtypes has been recognized as an important factor for the emergence of mosaic viral forms with unpredictable antigenic and biologic properties (14–18). Published reports provide evidence that recombinant HIV-1 strains also play a visible role in regional subepidemics (19,20).

HIV-1 subtype B has predominated in the spread of HIV-1 in most Latin American and Caribbean countries, including Brazil (21), but an increasing number of non-B infections (e.g., subtypes F, C, D, and E [22–29], B/F recombinant [14,16]. and mixed HIV-1 infections [30]) are being reported in these regions. In Brazil, subtype B is the most prevalent, but many Brazilian subtype B samples present a GWGR motif at the top of the V3 loop (B″) (22–24,29,31,32), whereas the GPGR motif is found in most North American and European strains. With the objective of establishing an effective system to monitor HIV-1 variability, a Brazilian Network for HIV Isolation and Characterization was created, and this paper presents results of this national collaborative effort.

MATERIALS AND METHODS

Brazilian Network for HIV Isolation and Characterization

The Brazilian Network for HIV Isolation and Characterization (BNHIC) is organized on a three-tier basis, including primary sites, a central reference laboratory, and secondary laboratories, paralleling the organization of the comparable UNAIDS program (24). The primary sites comprised the states of Minas Gerais, Rio de Janeiro, and São Paulo, which were responsible for the selection of volunteers, as well as collection and shipment of samples to the central laboratory at the Advanced Laboratory of Public Health, Centro de Pesquisa Gonçalo Moniz, Fundação Oswaldo Cruz in Salvador, Bahia. The central laboratory was responsible for HIV-1 isolation, expansion of viral stocks, and distribution of samples to secondary laboratories. Biologic, immunologic, and genetic characterization of HIV-1 strains was carried out by the secondary laboratories at AIDS and Molecular Immunology Laboratory, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro; Advanced Laboratory of Public Health, Centro de Pesquisa Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Bahia; Laboratory of Molecular Virology, Universidade Federal do Rio de Janeiro, Rio de Janeiro; Laboratory of Retrovirology and Microbiology Services, Adolfo Lutz Institute, São Paulo; and Virus Laboratory, Universidade Federal de Minas Gerais, Minas Gerais.

Patient Selection and Blood Collection

Blood samples were randomly collected from 35 HIV-seropositive patients at the AIDS Reference Center, Santos, São Paulo ($n = 8$); Providence Bank Outpatient Clinic, Rio de Janeiro ($n = 22$); and University of Minas Gerais Outpatient Clinic, Minas Gerais ($n = 5$). After signed informed consent was obtained, 40 ml ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood and 10 ml blood without anticoagulant were collected from each patient in Safety Monovette syringes (Sarstedt, Germany), and the samples were shipped at ambient temperature to the central laboratory within 24 hours of blood donation. Samples were labeled using the WHO/UNAIDS nomenclature system (33).

HIV serology was carried out by ELISA and Western blot. $CD4^+$ T cells were counted by flow cytometry (Epics XL, Coulter, FL, U.S.A.) at the Rio de Janeiro and Minas Gerais sites, and by Facscount (Becton-Dickinson, CA, U.S.A.) in São Paulo. Viral load was measured by nucleic acid sequence–based amplification procedure (NASBA, Organon, Holland) in plasma samples frozen at −70°C within 24 hours of blood collection.

Preparation of Complete Sets: Sample Processing, Virus Isolation, and Expansion

HIV-1 isolation and expansion were performed according to WHO/UNAIDS Guidelines (34). Viral growth was monitored by p24 antigen production (DuPont, Wilmington, DE, U.S.A.) every 3 or 4 days, and positive supernatants were saved and further expanded by infecting new batches of phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) from normal donors.

Biologic Characterization

Syncytium-inducing (SI) and non–syncytium-inducing (NSI) phenotypes were determined by infecting the T $CD4^+$ tumor cell line MT-2, as previously described (24,34). In brief, primary culture supernatants containing 5 ng/ml of p24 were incubated with 5×10^4 MT-2 cells in flat-bottomed microtiter plates, at 37°C, with 5% CO_2, and monitored for cytopathic effects every 2 or 3 days, under microscopic observation.

Genetic Characterization: HIV-1 Genetic Subtyping, Nucleotide Sequence, and Phylogenetic Analyses

DNA was extracted from uncultured PBMC using the proteinase-K sodium dodecyl sulphate method, followed by phenol/chloroform extraction, and amplified by nested polymerase chain reaction (PCR) using primers as described elsewhere (35,36). HIV-1 *env* genetic subtypes were also determined by heteroduplex mobility assay (HMA) using a HMA/HIV-1 subtyping kit (36). Brazilian subtype B variant (B″) was identified based on the restriction fragment length polymor-

phism (RFLP) on digestion with Fok I restriction enzyme (29,31,32). PCR products were directly sequenced, using ED31, ED33, and ES7 oligonucleotides as sequencing primers, and the dideoxy chain termination method (Sequenase Version 2.0, Amersham Life Science, Arlington Heights, IL, U.S.A.). Sequence data were analyzed using Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, MI, U.S.A.). For subtype determination, nucleic acid *env* C2-V3 sequences were trimmed to 345 nt and aligned with sequences representative of the HIV-1 group M subtypes available in the Los Alamos database (10). Alignments were generated using DNASIS version 2.1 software (Hitachi, Brisbane, CA, U.S.A.) and manually edited to introduce gaps to maintain alignments. Phylogenetic analysis was performed using the Phylip software package (University of Washington, WA, U.S.A.) (37). Evolutionary distances were estimated using Dnadist (Kimura two-parameter method), and phylogenetic relationships were determined using Neighbor (neighbor-joining method) (38). Reproducibility of branching patterns was assessed using Seqboot (bootstrap method; 100 replicates), and a consensus tree generated using Consense. SIV_{CPZgab} was used as the outgroup.

V3 Peptide Seroreactivity

V3 peptide seroreactivity was tested in an indirect enzyme-binding immunoassay (EIA) (39,40), using a panel of 14/15 meric biotinylated peptides, each based on consensus V3 sequences for the following HIV-1 subtypes: A (KSVHIGPGQAFYAT), B (NTRKSIHIGPGRAFY), B″ (NTRKSIHMGWGRAFY), C (KSIRIGPGQTFYAT), D (RQRTHIGPGQALYTT), F (RKSIHLGPQAFYTT), and F_{Br} (NTRKSIPLGPGRAFY). These peptides (with 70% purity) were purchased from Chiron Mimotopes (San Diego, CA, U.S.A.). Briefly, the EIA protocol was based on binding the biotinylated V3 peptides onto streptavidin-coated microtiter plates (Nunc, Roskield, Denmark), followed by the addition of 100 μl serially diluted plasma samples. The plates were incubated for 1 hour at 37°C, and overnight at 4°C. Bound antibodies were detected with peroxidase-labeled goat anti-human IgG conjugate and TMB/H_2O_2 substrate. A pool of normal human plasma was used as a negative control to determine cutoff values.

Neutralization Assay

Neutralization assays were run simultaneously with median tissue culture infective dose ($TCID_{50}$) titration, using the same donor PBMC and supernatants from expanded cultures as described before (41,42). Neutralization was evaluated by the reduction of HIV-1 p24 production (DuPont, Wilmington DE, U.S.A.) in the culture supernatants on day 7. Neutralization was considered positive when p24 production was reduced by >75% compared with the quadruplicate $TCID_{50}$ titration control wells of the same viral dilution. The inhibitory potency of the sera was determined through neutralization assay using the laboratory-adapted isolate MN.

Statistical Analysis

Fisher's exact test for two-sided tail value was used (GraphPad [In Stat] Software, San Diego, CA, U.S.A.). Statistical significance was defined as $p < .05$ values.

RESULTS

Patients: Demographic, Epidemiologic, Clinical, and Laboratory Data

Table 1 summarizes the demographic, epidemiologic, clinical, and laboratory data of the patients. The age distribution of patients ranged from 15 to 61 years (average, 37.4 years). Infection with HIV-1 occurred through homosexual ($n = 14$; 40%), bisexual ($n = 7$; 20%), and heterosexual ($n = 14$; 40%) activities. Most samples ($n = 30$) were obtained from asymptomatic individuals with $CD4^+$ T-cell counts ranging from 115 to 1191 cells/mm^3, and viral loads varying from <0.4 to 980 × 10^3 mRNA copies/ml. Five samples were obtained from AIDS patients. Patients 95BRRJ005, 95BRRJ009, 95BRRJ012, 95BRRJ022, and 95BRSP003 were receiving antiretroviral therapy at the time of blood donation.

HIV-1 Genetic Characterization: HIV-1 Genetic Subtyping and Nucleotide Sequence Analysis

The HIV-1 C2V3/*env* region was amplified from the PBMC of all 35 samples and used for HMA subtyping. Three different subtypes were found (Table 2): subtype B was predominant (29 of 35; 82.9%), followed by subtype F (5 of 35; 14.3%) and one case of subtype C infection (2.8%). No evidence for geographic or sexual bias for representation of subtypes was apparent, subtype B strains were found in 20 men and in 9 women, subtype F infections were detected in 4 men and 1 woman, whereas the only subtype C infection was found in a woman from São Paulo.

Nineteen subtype B samples and all non-B isolates were chosen for the C2V3/*env* sequence. Phylogenetic analysis (Fig. 1) was performed for a subset of 13 B and 5 non-B subtype sequences. The subtype assignments based on the C2V3/*env* sequences were in 100% agreement with HMA subtyping results (Table 2).The highest sequence heterogeneity in the C2V3 region was observed among subtype B isolates with an average divergence of 21% (range, 4%–33%), two times higher than for subtype F isolates (average, 10.5%; range, 3.8%–13.5%).

The crown of the V3 loop of the subtype B sequences (Fig. 2) revealed at least four distinct variants. The most prevalent variant had a GWGR tetrapeptide at the tip of the loop (B″), found in 8 of 19 analyzed, (42%), followed by the GPGR motif (7; 37%), common among North American and European subtype-B strains. GPGS (10.5%) and GFGR (10.5%) motifs were each also documented in 2 cases. All four subtype F samples had the GPGR motif, which distinguishes the Brazilian subtype F strains (F_{Br}) from those of the same subtype described in other geographic regions (43). The only subtype C isolate has a GPGQ tetrapeptide at the tip of the V_3 loop, which is conserved throughout subtype C (9,10).

HIV-1 Isolation and Biologic Characterization

The results of HIV-1 isolation and biologic characterization are shown in Table 2. Virus isolation efficiency

TABLE 1. *Epidemiologic, clinical, and laboratory data from Brazilian individuals selected in the three evaluation sites*

Patient no.	Gender	Age (y)	PTR[a]	Clinical status	CD4 count (cells/mm^2)	Viral load ($\times 10^3$ mRNA$^-$ copies/ml)
95BRRJ001	M	35	Hom	Asympt	670	24
95BRRJ002	M	28	Bi	AIDS	519	85
95BRRJ003	M	15	Hom	Asympt	ND[b]	190
95BRRJ004	M	61	Hom	AIDS	<50	980
95BRRJ005	M	58	Hom	AIDS	191	160
95BRRJ006	M	26	Hetero	Asympt	ND	260
95BRRJ007	F	30	Hetero	Asympt	673	<0.4
95BRRJ008	F	42	Hetero	Asympt	473	0.56
95BRRJ009	F	43	Hetero	Asympt	ND	<0.4
95BRRJ010	M	37	Hetero	Asympt	612	61
95BRRJ011	M	49	Bi	Asympt	1098	4.8
95BRRJ012	M	28	Hom	Asympt	330	77
95BRRJ013	M	45	Hetero	Asympt	1191	190
95BRRJ014	M	53	Bi	Asympt	115	370
95BRRJ015	M	32	Bi	Asympt	549	27
95BRRJ016	F	48	Hetero	Asympt	689	0.47
95BRRJ017	M	39	Hom	Asympt	275	800
95BRRJ018	F	42	Hetero	Asympt	1115	8.7
95BRRJ019	F	31	Hetero	Asympt	657	24
95BRRJ020	M	30	Hom	Asympt	553	42
95BRRJ021	M	38	Bi	Asympt	ND	860
95BRRJ022	M	41	Bi	AIDS	ND	8.2
95BRSP001	F	24	Hetero	Asympt	ND	280
95BRSP002	F	46	Hetero	Asympt	409	55
95BRSP003	F	56	Hetero	Asympt	258	<0.4
95BRSP004	F	21	Hetero	Asympt	303	20
95BRSP005	M	41	Bi	Asympt	328	22
95BRSP006	F	46	Hetero	Asympt	198	180
95BRSP007	M	41	Hom	Asympt	498	140
95BRSP008	M	35	Hom	AIDS	348	ND
96BRMG001	M	51	Hom	Asympt	599	250
96BRMG002	M	22	Hom	Asympt	344	130
96BRMG003	M	29	Hom	Asympt	351	55
96BRMG004	M	30	Hom	Asympt	1071	ND
96BRMG005	M	18	Hom	Asympt	708	43

[a] Presumed transmission route (PTR): Hom, Male homosexual; Bi, male bisexual; Hetero, Heterosexual.
[b] Not done.

was 88.6% (31 of 35). Three of four HIV-1 culture-negative samples were from patients with low viral load levels (<0.4, 0.47, and 0.56 $\times$ 10^3 mRNA copies/ml; Table 1). Samples from antiretroviral treated patients had viral load varying from <0.4 to 160 $\times$ 10^3 mRNA copies/ml and all were positive in HIV-1 coculture. Most cocultures (29 of 31) became positive in <9 days and reached peaks of p24 production by day 14, although replication patterns varied widely. Some isolates had a suggestive "rapid/high" replication phenotype, replicating quickly and to high titers in primary and secondary expansion cultures, reaching high levels of p24 production (range, 76–209 ng/ml). Of the isolates, 35% did not produce high levels of virus in primary or secondary expansion cultures, showing a suggestive "slow/low" replication pattern (24). No differences were evident between subtype B and non-B isolates in isolation efficiency and replication patterns. Of the isolates, 84% (26 of 31) had the NSI biologic phenotype on MT-2 cells, including 4 of 5 from AIDS patients. Five isolates had the SI phenotype (1 from an AIDS patient, 4 from asymptomatic patients). Three of the latter had relatively high CD4$^+$ T-cell counts (258, 498, and 612 cells/mm^3). However, a parallel was found between SI phenotype and virus replication patterns in vivo and in vitro, as indicated by high plasma viral load in three (61, 140, and 280 $\times$ 10^3 mRNA copies/ml) and high levels of HIV-1 p24 antigen production in culture (range, 120–209 ng/ml). All non-B isolates had the NSI phenotype and, in general, grew poorly in culture, releasing low levels of P24 antigens during the isolation procedure (data not shown). SI phenotypes of the cultured viruses were not associated with the presence of basic amino acids at positions 11, 24, or 25 of the V3 loop (24,44) (Fig. 2).

TABLE 2. *HIV-1 isolation, genotype, phenotype, seroreactivity, and neutralization from Brazilian study participants*

Patient no.	HIV isolation	Genotype[a]	Phenotype[b]	Seroreactivity[c]					Neutralization[d]	
				B	BRW	F	FBR	C	Aut (%)	MN (%)
95BRRJ001	Y	B″	NSI	–	++	+	+	+	NA	98
95BRRJ002	Y	B	NSI	+	+	+	++	+	48	34
95BRRJ003	Y	F	NSI	+	+	+	ns	–	NA	92
95BRRJ004	Y	B	NSI	+	+	+	+	–	0	8
95BRRJ005	Y	B	NSI	+	++	–	+	–	94	0
95BRRJ006	Y	B″	NSI	ns	++	–	–	–	0	84
95BRRJ007	N	B	NA	ns	+	+	–	–	NA	100
95BRRJ008	N	B″	NA	–	++	+	–	–	NA	100
95BRRJ009	Y	B″	NSI	+	+	+	+	–	0	99
95BRRJ010	Y	B″	SI	–	++	+	–	–	0	54
95BRRJ011	Y	B	NSI	++	+	–	+	+	NA	100
95BRRJ012	Y	B	NSI	+	–	+	–	–	NA	47
95BRRJ013	N	B″	NA	+	++	++	–	–	NA	83
95BRRJ014	Y	F	NSI	++	–	+	ns	–	NA	100
95BRRJ015	Y	B	NSI	++	–	+	+	+	NA	29
95BRRJ016	N	B″	NA	+	++	–	–	–	NA	100
95BRRJ017	Y	B″	NSI	–	++	–	–	–	52	100
95BRRJ018	Y	B	NSI	+	–	+	–	–	85	100
95BRRJ019	Y	B	NSI	++	–	+	–	–	81	100
95BRRJ020	Y	B	NSI	++	+	+	–	–	0	0
95BRRJ021	Y	F	NSI	+	–	+	–	+	92	100
95BRRJ022	Y	B	NSI	++	+	+	+	–	0	100
95BRSP001	Y	B″	SI	–	+	–	–	–	0	100
95BRSP002	Y	B	NSI	++	–	+	+	–	NA	100
95BRSP003	Y	B	SI	–	–	–	–	–	0	89
95BRSP004	Y	F	NSI	+	–	–	+	–	ND	0
95BRSP005	Y	B	NSI	+	+	+	ns	–	NA	83
95BRSP006	Y	C	NSI	–	–	+	–	++	NA	76
95BRSP007	Y	B	SI	+	–	+	–	–	50	100
95BRSP008	Y	B	SI	+	–	+	+	–	NA	100
96BRMG001	Y	B	NSI	+	+	+	+	+	95	23
96BRMG002	Y	B	NSI	+	+	+	+	–	NA	21
96BRMG003	Y	B	NSI	+	–	–	ns	+	90	11
96BRMG004	Y	B	NSI	ns	–	–	+	+	NA	23
96BRMG005	Y	F	NSI	+	–	–	++	+	100	73

[a] Evaluated by heteroduplex mobility assay, *Fok*I restriction fragment length polymorphism and C2V3 sequence.
[b] Evaluated by syncytium formation in MT2 cells.
[c] Seroreactivity (see Materials and Methods for peptide sequence): –, reactivity below 1:100; +, seroreactivity titer 1:100–1:400; ++, seroreactivity titer > 1:400.
[d] Percentage of neutralization of viral replication mediated by autologous patients' plasmas on primary virus or reference isolate MN.
Y, yes; n, no; NSI, non–syncytium-inducing; SI, syncytium-inducing; ns, nonspecific binding, removable by 8M urea washing; aut, autologous; NA, not available; ND, not done.

V3 Peptide Seroreactivity

The presence and specificity of antibodies to consensus V3 peptides representing five subtypes of HIV-1 (B/B″, C, F/F_{Br} as shown in Table 2, and peptides A and D, data not shown) were determined using plasma from all 35 subjects studied. Antibodies specific for the A peptide were detected in plasma samples from 95BRRJ015 and 96BRMG001, whereas antibodies with specificity for peptide D were not detected. Relative to the genotype characterization results, recognition of homologous peptides was detected in plasma from 85% (17 of 20) of B, 9 of 9 of B″, 5 of 5 of F (3 of 5 with peptide F and 2 of 5 with peptide F_{Br}), and the C plasma recognized the C peptide. However, 15 of 20 B plasma samples also recognized the F peptide and 5 of 5 F plasma samples reacted with the B peptide. Only 3 plasma samples had monospecific reactivity, all B″ plasma samples, recognizing only the B″ peptide, and another 5 B″ plasma samples had relatively higher anti-B″ than F peptide reactivity. All other plasma samples had at least dual reactivity and could not be serotyped.

Neutralization Analysis

Of 31 primary isolates, 18 were grown to sufficiently high titers ($TCID_{50\%}$ >20/ml) to have their susceptibility to neutralization determined (Table 2). Autologous neu-

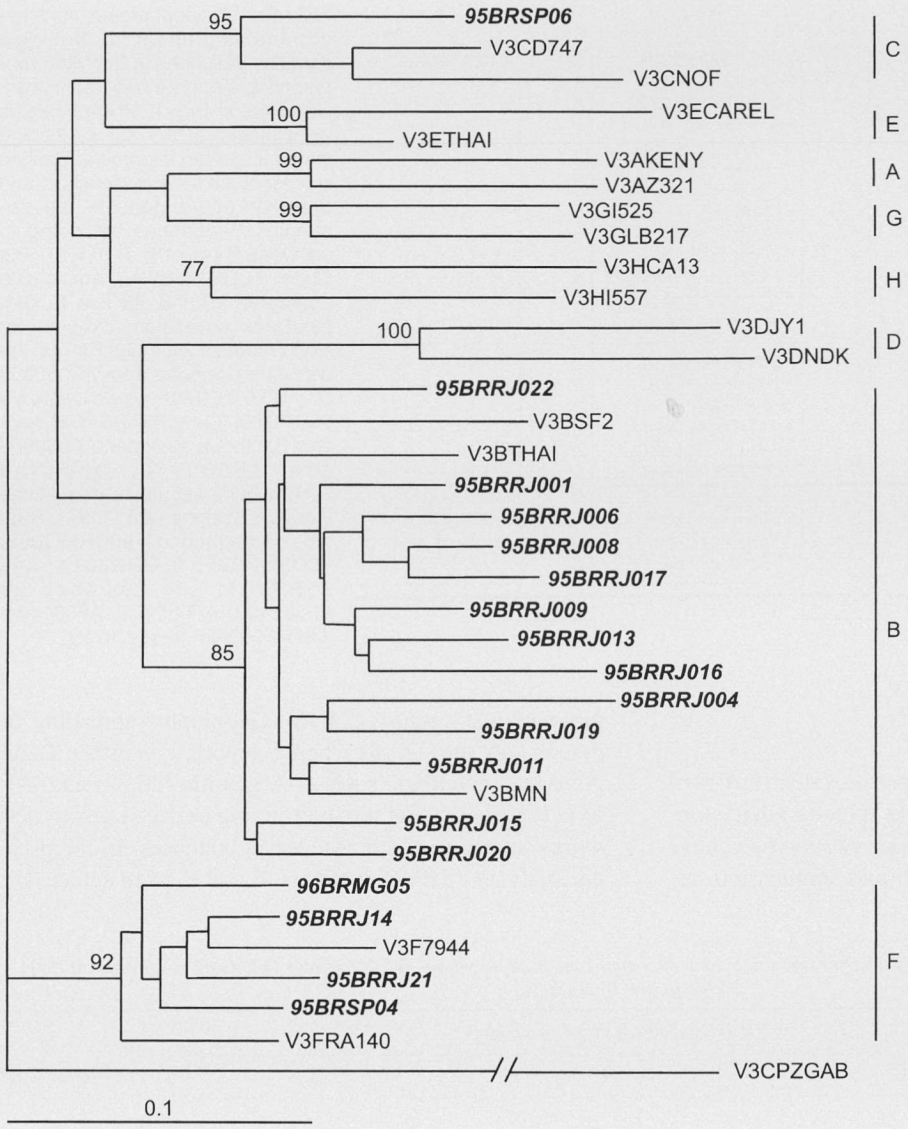

FIG. 1. Phylogenetic analysis of 18 representative Brazilian HIV isolates compared with 17 reference HIV-1 group M subtypes available in the Los Alamos database. Sequence SIV_{CPZgab} was used as outgroup. Aligned fragments were analyzed as described in Materials and Methods, and bootstrap values for 100 replicates are listed at the major subtype branches. Brazilian isolates sequenced in this study are highlighted and italicized. The hash mark on CPZgab indicates a truncation of actual distance. Accession numbers of the Brazilian sequences are specified in Figure 2. The scale bar represents a 10% divergence.

tralization was observed in 7 of 18 assays (39%) virus/plasma pairs, with lowest autologous neutralization for isolates from São Paulo (none of 3 versus 4 of 12 from Rio de Janeiro and all 3 from Minas Gerais). The Minas Gerais' plasma samples demonstrated the lowest potency in neutralizing the reference HIV-1 isolate MN (none of 5 versus 15 of 22 for Rio de Janeiro [$p = .0098$] or versus 7 of 8 for São Paulo [$p = .0047$]. The results obtained in the heterologous neutralization assays indicate a high cross-neutralization for B and F primary isolates, agreeing with the seroreactivity data (Table 3). In contrast, the results show a very low susceptibility of the B″ variant isolates to B and F plasma. Some plasma samples were unable to neutralize any primary isolates tested, neutralizing only the reference T-cell line–adapted MN isolate. The subtype C isolate did not grow to sufficient infectious titers to be evaluated by neutralization. However, the subtype C plasma was able to neutralize the only one B isolate tested and the reference HIV-1 MN isolate.

Comparison of heterologous neutralization results (Table 3) with V3 peptide serotyping (Table 2) indicated no consistent correlation. For example, although plasma 95BRRJ005 neutralized 2 clade B HIV-1 isolates and bound to the B synthetic peptide, plasma 95BRRJ012, although able to bind to the synthetic peptide, was unable to neutralize any 2 B isolates tested. A similar lack of correlation was observed for the B″ and F peptides.

		1	11		25	35
	V3 MN	CTRPNYNKRK	R	IHIGPGRAFYTTK	N	IIGTIRQAHC
	95BRRJ001	-----N-T--	G	--L-W-----A-G	E	---D----D-
	95BRRJ006	----SN-T--	S	-Q--W---L-A-G	E	---D----H-
	95BRRJ008	----SN-T--	S	-Q--W---L-A-G	E	---D------
	95BRRJ009	-----N-T--	S	--M-W------NG	E	---N------
	95BRRJ010	-----N-T--	S	--M-W------NG	E	---N------
	95BRRJ013	-----N-T-R	S	-PM-W--TL-A-G	E	---D------
	95BRRJ016	----TN-T--	G	--M-W--T----G	E	---D------
	95BRRJ017	-----N-T--	G	--M-W-----A-G	E	---D----Y-
	95BRRJ002	-----N-T--	S	--L----T--A-G	D	---N--L-Y-
B	95BRRJ005	-----N-T--	S	--L-------A-G	E	---NP-----
	95BRRJ015	-----N-T--	S	-P--------A-G	D	---D------
	95BRRJ019	-----N-T--	S	----------A-.	D	---D------
	95BRRJ020	-----N-T--	S	-T----------G	E	---N------
	95BRRJ022	-----N-T--	S	-QL-------A-G	E	---D--K---
	96BRMG003	-----N-T--	S	-T---------S.	D	---D----Y-
	95BRRJ004	-----N-T--	S	--F---S-M-A-G	Q	--.K------
	95BRRJ011	----SN-T--	S	------S---A-G	N	V--D------
	95BRRJ012	-----N-T--	S	--M-F---L-ATG	D	---D------
	95BRSP003	-----N-T--	S	-SM-F---L-A-G	E	---D------
	95BRRJ003	-----N-T--	S	--L----T--A-G	D	---N--L-Y-
F	95BRRJ014	-----N-T--	S	-QL-------A-G	E	---D--K---
	95BRRJ021	-----N-T--	S	--L---------G	E	---D--K---
	95BRSP004	-----N-T--	S	-HI-------A-G	E	---D--K---
	96BRMG005	-----N-T--	S	-P----------G	D	---D--K---
C	95BRSP006	-----N-T--	S	-RI---QT--A-G	-	---D------

FIG. 2. V3 loop amino acid sequence alignment of the new samples described in this work representing the three geographic vaccine sites in Brazil. MN sequences were placed at the top as leading sequence of the alignment. Dashes represent amino acid identities, and dots represent gaps in the sequence. Sequences belonging to subtypes B (n = 19), F (n = 5), and C (n = 1) are indicated. Amino acid positions 11 and 25 are boxed. GenBank accession numbers for HIV-1 sequences from Rio de Janeiro are assigned from AF060952 to AF060968; sequences 95BRSP003 and 95BRSP006 from São Paulo are assigned AF060969 and AF060970; and the 96BRMG003 sequence from Minas Gerais is assigned AF060971. Subtype F sequences 96BRMG005, 95BRSP004, 95BRRJ014, and 95BRJ021 are assigned as AF062422, AF062423, AF062424, and AF062425, respectively.

DISCUSSION

The HIV-1 subtype B is the most prevalent in Brazil and, along with subtype F, is widely spread in that country (21,23,24,29,45). Until now, cases of subtype C have been detected only in the southern and southeastern regions of that country (24,46). Geographic spreading of non-B subtypes is also being reported in other Latin American countries, such as Argentina (26,27) and Bolivia (28), indicating the intermixing between sexual networks in various Latin American countries. In the present study, two HIV-1 subtypes, B and F, were detected in

TABLE 3. *Comparison of heterologous neutralization activities of some of the plasma against HIV-1 isolates belonging to subtypes B or F including the B″ variant*

	HIV-1 Isolates/subtype or variants			
Plasma[a]	B	B″	F	HIV-1MN[b]
B				
95BRRJ005	89% 95BRRJ002,[b] 100% 96BRMG001	0% 95BRRJ006	6% 95BRRJ021, 23% 96BRMG005	0%
95BRRJ012	46% 95BRRJ018, 12% 95BRRJ019	38% 95BRRJ017	97% 95BRRJ021	47%
95BRRJ019	79% 95BRRJ018, 53% 95BRRJ020	85% 95BRRJ017	93% 95BRRJ021	100%
95BRSP002	16% 95BRSP003	3% 95BRSP001		100%
95BRSP005	0% 95BRSP003, 21% 95BRSP007	38% 95BRSP001		83%
96BRMG002	93% 96BRMG001, 19% 96BRMG003		100% 95BRRJ021	21%
B″				
95BRRJ006	100% 95BRRJ004, 100% 96BRMG001		35% 95BRRJ021	84%
95BRRJ009		0% 95BRRJ010	81% 96BRMG005	99%
95BRRJ010		0% 95BRRJ009	23% 95BRRJ021	54%
95BRRJ017	50% 95BRRJ018	56% 95BRRJ017	16% 95BRRJ021	100%
95BRSP001	5% 95BRSP003, 17% 95BRSP007			100%
F				
95BRRJ003	98% 95BRRJ02, 100% 95BRRJ004		87% 95BRRJ021	92%
95BRRJ014	84% 96BRMG01, 100% 96BRMG003		100% 96BRMG005	100%
95BRRJ021	75% 95BRRJ019, 40% 95BRRJ020	0% 95BRSP001	100% 96BRMG005	100%
96MGMG005	100% 96BRMG001, 80% 96BRMG003			73%
C				
95BRSP006	75% 95BRSP007			76%

[a] Plasma of patients harboring HIV-1 subtypes B or F or C.
[b] Percentage of viral neutralization mediated by the heterologous plasmas.

three vaccine evaluation sites, whereas the C subtype was detected only in São Paulo. In addition, HMA results were 100% concordant with subtype assignment based on proviral C2V3/*env* sequences, confirming reliability of HMA for molecular epidemiologic studies (24). It should be taken into account that the HMA/sequence strategy based on the C2V3/*env* region prevents detection of recombinant virus. Thus, to search accurately for recombination other regions of the viral genome should be analyzed. The highest sequence heterogeneity in the C2V3/*env* region was observed among subtype B viruses with an average dispersion of 21%. An increase of intrasubtype B heterogeneity over the past 5 years could be seen when compared with data (13.5%) from a previous study conducted in Brazil (10,23). Although the Brazilian B″ variant sequences could be segregated in a separate branch in the phylogenetic analysis, the intrasubtype heterogeneity of subtype B strains did not show any significant difference in the diversity among B″ and B isolates, suggesting that both variants emerged in Brazil at approximately the same time. The overall genetic diversity of subtype F isolates was nearly half as low (10.5%) as the overall diversity of subtype B strains (21%), indicating a later introduction of the F subtype in Brazil. Amino acid sequence analysis of HIV-1 isolates in this study showed a large prevalence (42%) of variant subtype B (B″) strain with the GWGR tetrapeptide. The GPGR sequence, characteristic of the North American subtype B, was also frequently found, in 37% of cases. Similar proportion between the two subtype B variants was reported in previous studies (22–24,29,31–32), suggesting that the frequency and proportion between the two subtype B variants has been maintained over time. These data are in contrast with the situation described in Thailand, where a rapid shift between the original B strains and newly emerging local variants with a different V3 loop (GPGQ motif) was observed (10,47–48).

Indeed, the origin of the GWGR variant in Brazil is not yet clear. This variant was first reported in 3 of 29 isolates from Japan (49), one of which was obtained from a woman who had emigrated from South America. However, based on the average dispersion (20%) of the B″ isolates documented in the present study, this variant seems to have been present in Brazil since the beginning of the AIDS epidemic in that country.

All subtype F sequences described in our study contained the GPGR motif. The GPGQ signature sequence, occasionally observed in Brazilian F viruses and in all Romanian F viruses (43), was not found in the subtypes we studied, with the exception of subtype C, in agreement with previous descriptions of this subtype in Brazil (24).

The V3 peptide seroreactivity studies have shown a very close antigenic relation and an extensive cross-reactivity between subtypes B and F, similar to the previously reported cross-reactivity between subtypes A and C (24,39,50). These observations confirm that there is no strict correlation between genetic subtypes and V3 serotypes, at least using the current V3 peptides and assay format.

Biologic characterization results demonstrated that primary isolates of HIV-1 exhibit a broad range of biologic variability. Even though it was not possible to correlate genetic subtypes with biologic phenotypes, several interesting observations could be made. First, all cases of non-B infections described here were associated with NSI phenotypes. The subtype B isolates included most NSI strains; all SI viruses were found within subtype B group. These results corroborate that HIV-1 isolates with an NSI biologic phenotype are the most frequently transmitted viruses, independent of their genetic subtype. Second, HIV-1 isolates with the NSI phenotype can vary broadly with regard to their replication capacity, because all NSI isolates were characterized by unique replication patterns both in vivo and in vitro. In addition, regarding to the coreceptor usage, preliminary results demonstrated that most of them use CCR5 as the cofactor for infecting target cells (51). Additional studies, including recently developed technologies to analyze the role of host genetics and sensitivity to chemokines, will be useful to better characterize these isolates (7). An association between NSI biologic phenotype and the absence of basic amino acids at positions 11 and 25 was verified. However, positive amino acids at those positions were not seen in either of two SI isolates sequenced.

The HIV-1 neutralization experiments confirm the previously reported data about the lack of correlation between genetic subtypes and neutralization serotypes (52,53). Subtypes B and F appear to be very closely related immunologically. However, our present data indicated low susceptibility of the variant B″ isolates against plasma samples from clades B and F. These data do not corroborate previous results. Indeed, we earlier demonstrated similar levels of susceptibility of B isolates against B and B″ plasma (42). The discrepancy in results could be due to the small number of samples analyzed in the current study. These findings suggest that intrasubtype B variability, specifically in the V3 region, is not a major obstacle for HIV neutralization, given that cross-neutralization patterns could be observed between HIV-1 isolates belonging to different genetic variants of subtype B strains in Brazil (42). To confirm that the HIV-1 intrasubtype and intersubtype genetic variability should not represent an insurmountable problem for HIV vac-

cine protection, more extensive cross-neutralization studies are in progress using stored biologic material from individuals participating in the present study.

Finally, this study has demonstrated the feasibility of the BNHIC. Future directions for further development of the Brazilian Network should include epidemiologic surveillance to encompass the entire geographic area of Brazil, studies of association of HIV-1 subtypes with modes of transmission and risk factors, and the establishment of a surveillance system to monitor the emergence of drug-resistant strains. This strategy would provide the most beneficial progress in the extremely complex area of HIV/AIDS-related research.

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