

## V3 Region Polymorphisms in HIV-1 from Brazil: Prevalence of Subtype B Strains Divergent from North American/European Prototype and Detection of Subtype F

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### ABSTRACT

Viral DNA sequences were determined over the V3 region of *env* from 28 infected individuals living in the high HIV-1 prevalence Brazilian cities of Rio de Janeiro and São Paulo. Twenty-six belonged to envelope sequence subtype B, prevalent in North America and Europe, and one was classified as subtype F, found recently in Brazil and in Romania (one appeared to be a B/F recombinant). Octameric sequences at the tip of the subtype B V3 loops were variable and distinct from those prevalent in North America and Europe. The GPGR motif, prevalent in North American/European strains, was found in only 8 (28.5%) sequences, whereas GWGR was found in 12 (43%) and novel sequences in 8 (28.5%). Brazilian subtype B sequences also diverged from the consensus North American/European strains over the remainder of the V3 loop. These results suggest that Brazilian HIV-1 B strains may have important antigenic differences from prototype subtype B strains currently being evaluated for use in HIV vaccines. These results should be taken into account for future vaccine programs in Brazil.

### INTRODUCTION

**H**UMAN IMMUNODEFICIENCY VIRUS type 1 (HIV-1) isolates have been found to differ in terms of replication rate, cell infectivity, cytopathicity, and cellular tropism. Some of these biological properties seem to be correlated with *in vivo* pathogenesis of HIV-1.<sup>1-3</sup> HIV-1 *in vivo* has also been shown to consist of a population of related genomes that can change with time.<sup>4</sup> Serologically defined subtypes of HIV-1 have been reported,<sup>2</sup> and seven sequence-defined subtypes have been defined that differ by up to 35% in the surface (SU) coding portion of the *env* gene.<sup>5</sup> In contrast, the diversity within an

individual usually ranges up to no more than 7%.<sup>5,6</sup> The importance of this genetic variation in terms of pathogenesis and vaccine development has been discussed by Wolfs *et al.*<sup>7</sup>

The highly variable V3 region of the envelope glycoprotein gp120 is functionally important in that it (1) is a determinant for T cell and macrophage cellular tropism,<sup>8-10</sup> (2) is involved in determining syncytium induction phenotype and replication rate,<sup>11,12</sup> (3) contains the epitope recognized by antibodies that neutralize HIV infection *in vitro*, the so-called principal neutralizing determinant (PND),<sup>13-16</sup> and (4) stimulates a potent immune response by cytotoxic and helper T lymphocytes.<sup>17-19</sup> Because of its biological functions and the role it may play in the

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induction of a protective immune response, this region of the envelope protein has been considered to be important for inclusion in vaccines.

Although Brazil ranks third to fourth in the world in the absolute number of reported AIDS cases, information is scarce with regard to the molecular diversity of Brazilian HIV-1 strains,<sup>20,21</sup> and the relationship of Brazilian sequences to those found in other parts of the world. Serological studies using peptides corresponding to the V3 region of different HIV-1 strains showed a lower reactivity of sera from Brazil against the prevalent North American/European MN strain than was seen with sera from North America, Europe, and even Africa.<sup>22,23</sup>

We have evaluated the degree of polymorphism among Brazilian HIV-1 genomes by sequencing the *env* V3 region, amplified by polymerase chain reaction (PCR) directly from peripheral blood mononuclear cells (PBMCs) of HIV-1-infected people living in Rio de Janeiro and São Paulo, cities of high incidence of HIV-1 infection. We have observed significant

differences between the V3 sequences in Brazilian HIV-1 and those in the North American/European envelope protein currently in development for use as recombinant vaccines. The differences reported here may represent potential constraints on the effectiveness of these recombinant vaccines in Brazil.

## MATERIALS AND METHODS

### Patient population

HIV-1-infected individuals belonging to the cohort of patients enrolled in a multicentric study of heterosexual transmission of HIV in Rio de Janeiro ( $n = 28$ ), and four patients from São Paulo attending the AIDS clinic at the Adolpho Lutz Institute, were included in this study (Table 1). Samples were obtained in 1990, 1991, and 1992. All patients were serologically positive as confirmed by enzyme-linked immunosorbent assay (ELISA),

TABLE 1. SUMMARY OF EPIDEMIOLOGICAL AND LABORATORY DATA FOR BRAZILIAN SUBJECTS

Subject <sup>a</sup>	Year	Risk group <sup>b</sup>	Sex	CDC clinical class	CD4 <sup>+</sup> cells (per mm <sup>3</sup> )
RJ 12	1990	Heterosexual	F	II	444
RJ 14	1990	Bisexual	M	II	NA <sup>c</sup>
RJ 17	1990	Blood product	M	II	563
RJ 19	1990	Blood product	M	II	697
RJ 27	1990	Bisexual	M	IV	090
RJ 33 <sup>d</sup>	1990	Heterosexual	F	III	332
RJ 49	1990	Heterosexual	M	III	1279
RJ 54	1990	Heterosexual	F	II	684
RJ 59	1990	Heterosexual	F	II	433
RJ 62	1990	Heterosexual	F	II	704
RJ 64	1990	Heterosexual	F	NA	204
RJ 70	1990	Bisexual/IVDU	M	IV	635
RJ 370 <sup>d</sup>	1991	Bisexual	M	II	503
RJ 379	1991	Heterosexual	F	II	657
RJ 477	1992	Heterosexual	M	II	981
RJ 478	1992	Heterosexual	F	II	401
RJ 483	1992	Heterosexual	M	NA	496
RJ 484	1992	Heterosexual	F	NA	247
RJ 485 <sup>d</sup>	1992	Heterosexual	F	II	494
RJ 491 <sup>d</sup>	1992	Bisexual	M	III	965
RJ 623	1992	Heterosexual	F	II	799
RJ 625	1992	Heterosexual	F	NA	311
RJ 626	1992	NA	M	II	590
RJ 636	1992	Heterosexual	F	IV	015
RJ I01	1992	Heterosexual	F	II	1031
RJ I02	1992	Heterosexual	F	II	NA
RJ I03	1992	Heterosexual	F	II	NA
RJ I04	1992	Heterosexual	F	II	550
SP 1	1992	Homosexual	M	II	>500
SP 2	1992	Homosexual	M	II	>500
SP 3	1992	Homosexual	M	II	>500
SP 4	1992	Homosexual and blood product	M	IV	200

<sup>a</sup> RJ, Rio de Janeiro; SP, São Paulo.

<sup>b</sup> Heterosexual females, sexual partners of HIV-seropositive individuals; heterosexual males, sexual intercourse with prostitutes. IVDU, Intravenous drug user.

<sup>c</sup> NA, Not available.

<sup>d</sup> Samples not amplified with *env* primer sets.

immunofluorescence assay (IFA), and Western blot tests, and were scored according to Centers for Disease Control (CDC) clinical classification. CD4<sup>+</sup> cell evaluations were done by flow cytometry.

#### DNA preparation from peripheral blood mononuclear cells

Genomic DNA was extracted by the proteinase K-sodium dodecyl sulfate (SDS), organic extraction method from 10<sup>6</sup> PBMCs purified by Ficoll-Hypaque density centrifugation. Briefly, pelleted cells were resuspended in 200 ml of 50 mM Tris-HCl (pH 7.8), 10 mM ethylenediaminetetraacetic acid (EDTA), containing 1% SDS and proteinase K (100 mg/ml), incubated at 37°C overnight, extracted with phenol (v:v), phenol-chloroform, and chloroform, and then ethanol precipitated. The samples were resuspended in 20 ml of water and the concentration of DNA evaluated by agarose gel electrophoresis in comparison to a known concentration of DNA.

#### Polymerase chain reaction amplification of HIV-1 *env* sequences

DNA (200 to 500 ng) from each sample was used as template for the amplification of a region of the *env* gene including a portion of C2 through V3, V4, and V5, using a nested protocol.<sup>24</sup> In the first round, primers ED3 (5' TTAGGCATC-TCCTATGGCAGGAAGAAGCGG) (positions 5956–5986 of the HXB2 genome) and ED12 (5' AGTGCTTCCTGCTGCTC-CCAAGAACCCAAG) (positions 7792–7822) were used. Aliquots (5 ml) of the first PCR round were reamplified using ES7 (5' TGTAACAACGACGGCCAGTCTGTAAATGGCAGTCTAGC) (positions 7001–7021) and ES8 (5' CAGGAAACAGC-TATGACCCACTTCTCCAATTGTCCCCACA) (positions 7646–7667) primers. The PCR conditions consisted of 3 cycles at 97°C (1 min), 55°C (1 min), and 72°C (2 min), followed by 32

#### Amplification and cloning of HIV-1 DNA from peripheral blood mononuclear cell DNA

DNA of sample RJ IO3, which could not be amplified with the above set of *env* primers, was successfully amplified using ED3 (5' TTAGGCATCCTATGGCAGGAAGAAGCGG, positions 5956–5985 of the HXB2 genome) and ED14 *env* primers (5' TCTTGCCTGGAGCTGCTTGATGCCCCAGAC, positions 7931–7960 of the HXB2 genome) in the first round (using the same buffer as in the first round described above). For the second round, the J-5'-2KSI and J-3'-2-KSI-2 primers of Wolfs *et al.*<sup>26</sup> (5' caucaucaucauGCAGUCUAGCAGAAGAAGA and 5' cuacuacuacuaUUCUGGGUCCCCUCCUGAGGA [positions 7011–7029 and 7333–7313 on HXB2, respectively]) were modified for use with the Clone-Amp kit (GIBCO-Bethesda Research Laboratories [BRL], Gaithersburg, MD). The amplification buffer contained 3 mM MgCl<sub>2</sub>. The resulting PCR fragment contained the V3 loop and flanking regions, and was digested with uracil DNA glycosylase to cleave uracil-containing primers, annealed with pAMP1 vector DNA (GIBCO-BRL), and transformed into *Escherichia coli* DH5α cells (GIBCO-BRL). Plasmid DNA from a miniprep derived from an individual colony was prepared using a Qiagen-tip 20 column (Qiagen, Chatsworth, CA), and sequenced with Sequenase version 2.0 (United States Biochemical).

#### Nucleotide sequence analysis

Direct sequencing of HIV-1 PCR product without cloning yielded a consensus nucleotide sequence of the predominant V3 loop and flanking regions for all samples except RJ IO3. The RJ IO3 sequence was derived from a single provirus. Nucleotide and predicted amino acid sequences were aligned with each other and with a set of available envelope subtype B HIV-1 isolates,<sup>5</sup> using the program MASE.<sup>27</sup> A phylogenetic tree was constructed using the PHYLIP 3.5c package of computer programs.<sup>28</sup> The branching order was determined using DNADIST

### Polymerase chain reaction of env DNA from Brazilian patients

A region of the viral envelope gene (*env*) consisting of the V3 loop with flanking regions was PCR amplified directly from PBMC DNA and the product sequenced without subsequent cloning. This yielded the majority sequence present within the PBMCs of 27 of 32 individuals. The remaining 5 DNA samples could not be PCR amplified using the first set of nested primers specified in Materials and Methods. Proviral DNA from the five remaining negative samples, were, however, successfully amplified (data not shown) using a nested set of primers specific for the first exon of the *tat* gene,<sup>29</sup> suggesting that viruses within these individuals might be divergent at primer annealing sites within *env*. Indeed, the *env* gene of one of these remaining sequences, sample RJ IO3, could be PCR amplified using a different nested set of *env*-specific primers (see Materials and Methods). Insufficient DNA was available from the other four samples (RJ 33, RJ 370, RJ 485, RJ 491) to allow amplification with the second set of *env* specific primers.

### Definition of subgroup B and F sequences in Brazilian samples

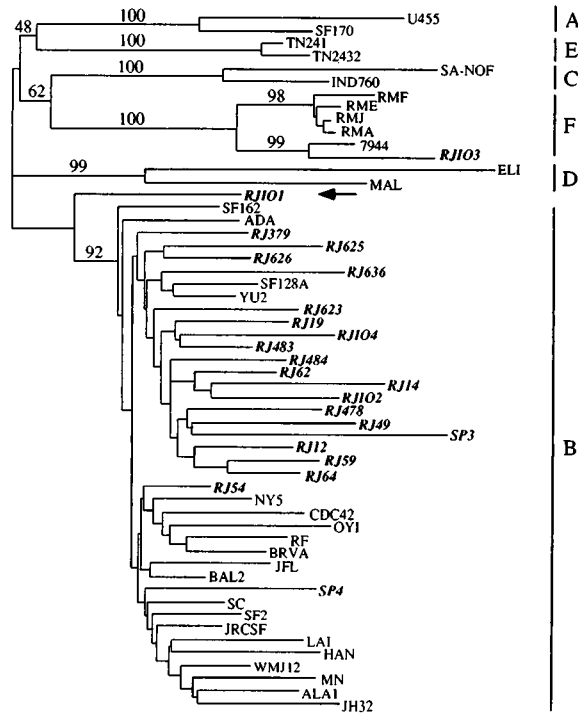
The alignment of the deduced amino acid sequences from the 28 Brazilian samples evaluated in this study is presented in Fig.

1. Twenty-two of the 28 samples were sequenced over the entire V3 region shown, whereas partial sequences from the remaining 6 samples were obtained. The average nucleotide diversity among a subset of these sequences (excluding RJ IO1 and RJ IO3) in the region shown in Fig. 1 is 13.5%, and ranged between 7.3 and 21.4% for all pairwise comparisons. Some positions at the tip of the V3 loop and the flanking region downstream from the V3 loop were highly divergent, with three or more different amino acids in those positions.

Although six of the Brazilian samples analyzed were not sequenced for the entire region shown in Fig. 1, enough sequence was obtained to determine that, with the exceptions of RJ IO1 and RJ IO3, all samples belong to subtype B. Divergence from the published subtype B sequences included in Fig. 2 ranged from 6.3% (RJ 54 vs. JRCSF) to 20.7% (SP 3 vs. CDC42). The RJ IO3 sequence was distinct in that it showed a 22.5 to 28.4% divergence from the subtype B Brazilian sequences, and a 23.6 to 31.7% difference from representative sequences of the A-E subtypes included in Fig. 2. However, RJ IO3 was only 8.3% divergent from HIV sequence 7944 described in Brazil<sup>21</sup> and 11.8 to 14.9% divergent from several characterized sequences from Romanian children<sup>30</sup> (Figs. 1 and 2). Therefore, RJ IO3 and the unique 7944 sequence from Brazil described by Potts *et al.*<sup>21</sup> can be classified as members of the newly identified HIV-1 envelope sequence subtype F.<sup>5,24,30-32</sup> Interestingly, the differences between RJ IO3 and other Brazil-

	<--	V3 loop	-->
BR-CONS	VVIRSENFNTNAKTIIVQLNESVEINCTRPNNTRKSIHIGWG-RAFYATGEIIGDIRQAHCNLSRAKWNTLKRQIAIKLREQF--NKTIV-FNQ		
RJ12	I.....L.....K.V.....S.....G.....I.....A.....K.E.....E.....K.....		
RJ484	..L.....D.V.....S.....G.....T.....K.Y.....I.....T.....E.....R.....E.....K.....I.....		
RJ14	..V.....I.....H.....T.....V.....L.T.....N.....I.G.T.....G.....W.....A.....K.....K.....Q.F		
RJ59	.....G.....L.....A.....T.NKT.EK.E.V.....V.....T.....		
RJ64	.....L.....T.Q.E.....EK.....G.VI.....		
RJ62	..V.....I.....S.....L.....V.....E.....T.N.....E.....K.....		
RJ70	.....S.....S.....L.....T.....		
RJ478	.....K.....Q.....M.....HTN.....N.....GIE.N.....T.....E.....G.....I.....T.....		
RJ49	.....I.....V.....S.....M.....T.....V.....ED.....EK.....R.....H.K-E.P.....K.....		
SP1	I.....H.....V.....S.....M.....R.....A.NS.....R.....		
SP2	I.....K.....D.T.I.....T.....M.....T.D.....I.....		
SP3	I.....K.....SD.....T.K.D.I.....G.M.....T.....R.....I.KVA.NR.GR.....N.....T.....IK.....		
RJ477	.....I.....V.....V.....R.....M.L.....D.....YX.....		
RJ483	I.....V.....G.M.L.....G.V.....I.....R.....K.....K.....N.....		
RJIO2	I.V.....I.....V.V.....I.....I.ETR.....EK.....K.....V-V.....K.S-Q.....		
RJIO4	.....I.....H.....G.....F-TLF.A.....I.....N.A.TK.M.K.....S.....K.....		
RJ19	G.....D.....I.....QY.T.GG.....V.....I.KE.N.....T.K.....		
RJ17	.....V.....M.P.....Y.T.....L.....A.....		
RJ27	.....D.....D.....L.....A.....R.....P.....GG.INKEN.....		
RJ379	.....D.....D.....I.....V.....G.....P.....T.....D.....ND.R.....NGTE.I.....		
RJ54	.....I.D.....V.....P.....T.....Q.N.....L.VT.....G.....A.....S.....		
RJ623	..V.....D.....I.....T.....S.....R.....L.P.-G.....T.....N.K.F.T.NGT.E.....A.....K.....		
RJ625	.....D.V.....T.....P.....WF.R.GTT.....ST.N.....V.....Q.....G.....A.I.....K.....		
RJ626	.....IA.....R.P.-S.....D.....T.EH.T.GR.Q.....N.....I.....		
RJ636	G.....I.....Q.V.....P.AP.-S.WF.....K.VH.E.....K.....E.....Q.....		
SP4	.G.....D.....M.....TP.....Y.....RKS.P.....T.....V.....KR.ND.I.VTE.H.K.....		
NA-CONS	.....D.....P.....T.....I.....N.....VT.....G.....		
RJIO1	II.....O.ISD.....I.....H.....N.P.....D.....Y.I.O.N.E.....G.....K.....K.A-T.....		
RJIO3	II.SFQ.ISD.....HF.....Q.D.....I.....PL.P.....K.....KVHGTQ.NKM.E.VKAT.QSH.S-A.K-TS.....		
7944	.Q.ISD.....H.....Q.T.....QL.P.....T.....K.....VNGSQ.NK.E.VRAE.GSH.P-A.K-T.S.....		
RMA	Q.ISD.T.....H.....T.Q.....L.P.-Q.....D.....K.....VNGTQ.N.VQRVRA.SH.P-T-S.....		
RME	Q.ISD.T.....H.....T.Q.....L.P.-Q.....D.....K.Y.V.GTQ.N.VQRVRA.KSH.P-T-T.....		
RMF	Q.ISD.T.....H.....T.Q.....L.P.-Q.....T.D.....K.....V.GTQ.N.VHRVRE.QPL.P-R.G-S.....		
RMJ	Q.ISD.T.....H.....T.Q.....L.P.-Q.....D.....K.....V.GTQ.N.VQRVRA.QSH.P-T-S.....		

**FIG. 1.** The alignment of Brazilian, B subgroup consensus HIV-1 and F subgroup sequences in the V3 region. The putative recombinant sequence RJ IO1 is shown between B subgroup sequences above it and F subgroup below. Consensus sequences were generated separately for all Brazilian and North American/European B subtype sequences shown in Fig. 2. Amino acids identical to the Brazilian subtype B consensus sequences are shown as dots. Four most divergent sequences from Romanian children<sup>30</sup> and F subtype sequence 7944 from Brazil<sup>21</sup> (GenBank accession numbers L19571, L19575, L19576, L19579, and L19237, respectively) are also shown.



**FIG. 2.** Phylogenetic tree for 22 Brazilian HIV-1 sequences described in this article (shown in bold italics), 20 subtype B sequences, 5 subtype F sequences, and representative sequences from other HIV-1 subtypes. The tree was generated for the fragment of the envelope gene shown in Fig. 1, excluding the first 15 nucleotides not sequenced in the Romanian samples.<sup>30</sup> Only completely sequenced Brazilian C2V3 sequences were included. Phylogenetic analysis was done using the PHYLIP programs<sup>28</sup> (see Materials and Methods). The numbers near the major branches show how many times out of 100 bootstrap repetitions the sequences to the right clustered together (putative recombinant virus RJ IO1 was excluded from bootstrap analysis). HIV-1 isolates are from the following countries: all B subtype from the United States, except OYI (accession number M26727) from Gabon and HAN<sup>5</sup> from Germany; 7944 from Brazil; ELI (K03454) and MAL (K03456) from Zaire; RM from Romania; SA-NOF (L07426) from South Africa; IND from India (5); TN from Thailand (L03700 and L03703); U455 (M62320) from Uganda; and SF170 (M66535) from Rwanda.

ian sequences were mainly localized in the regions flanking the V3 loop rather than within the loop itself (Fig. 1).

In the phylogenetic analysis shown in Fig. 2, the 22 longest Brazilian sequences were analyzed together with HIV-1 *env* sequences representing subtypes A–E<sup>5</sup> and subtype F. This analysis confirms that the Brazilian sequences (shown in bold italics) belong to the B and F subtypes with B predominating.

The N-terminal region of the RJ IO1 sequence shown in Fig. 1 seems to match the subtype F sequences better than the subtype B sequences. However, the remaining part of the sequence is more similar to subtype B sequences. There are four amino acid changes (Q.IS.H in Fig. 1) and two silent mutations (not shown) present in the N-terminal region of RJ IO1 and all of the subtype F sequences in Fig. 1, which are either not present, or are rare, in

the corresponding region of subtype B sequences. The potentially recombinant origin of RJ IO1 is also evident in Fig. 2, where it is positioned as an intermediate between B and other subtypes. Additional sequencing of RJ IO1 and RJ IO3 is in progress to address the hypothesis that RJ IO1 is a recombinant between B and F HIV-1 subtypes. It is noteworthy that the one subtype F sequence and the putative B/F recombinant sequence (RJ IO3 and RJ IO1, respectively) were obtained from recent seroconverters.

*Differences between Brazilian and North American/European subtype B V3 loop sequences*

Only 23% of the 26 subtype B Brazilian sequences analyzed had the GPGR motif in the tip of the V3 loop that, in contrast, is conserved within 65% of the North American/European viruses.<sup>5</sup> The Brazilian subtype F sequences RJ IO3 and 7944<sup>21</sup> and the putative recombinant sequence RJ IO1 all had the GPGR motif, whereas the Romanian subtype F sequences had GPGQ. The Romanian sequences also had other characteristic amino acids outside of this motif (Fig. 1). The GWGR tetrapeptide was present in 43% of the Brazilian samples analyzed, whereas 29% had different motifs, such as GLGR, GPGG, and GGGR, among others. The frequency of variants in the octameric sequence at the tip of the V3 loop from Brazilian HIV-1 samples was compared to 159 North American/European subtype B samples<sup>5</sup> in Table 2. Eighteen of 28 Brazilian samples analyzed (64%) were unique to Brazil, including the octamer HLGW-GRAF, which was present in 4 of 28 samples analyzed (14%). This demonstrates significant amino acid differences between Brazilian and North American HIV-1 envelopes in this immunologically important epitope.

A comparison of the 35 amino acids corresponding to the V3 loop between Brazilian subtype B HIV-1 samples and some prevalent North American/European HIV-1 strains under evaluation for use in vaccine development showed that the level of conservation with the LAI (former BRU) strain was low, ranging from 56 to 75% (mean, 66%). For the prevalent MN strain V3 loop, the conservation with Brazilian samples ranged from 64 to 83% (mean, 71%) whereas higher levels were obtained when compared to the SF2 or SC strains (71 to 91%; mean, 79.5%). The similarity values between the North America/European consensus sequence and these various strains are on average higher (75, 83, 89, and 89%, respectively).

*Sequencing of tissue culture isolates*

Comparison of the amino acid sequences obtained by direct sequencing of PCR product from PBMCs with those obtained after a single PBMC cocultivation passage<sup>32</sup> yielded similarities of 96 and 93% for sample pairs from subjects RJ 623 and RJ 625, respectively (data not shown). This suggests that the sequences obtained directly from PCR products of PBMC proviruses are highly related to the sequences of replication-competent viruses obtained after a single passage in PBMCs. Primary isolates recovered after cocultivation from nine Brazilian PBMC samples<sup>33</sup> will be useful for the evaluation of antigenic variability of Brazilian HIV-1 strains in functional assays such as viral neutralization.

TABLE 2. FREQUENCY OF V3 LOOP OCTAMERIC TIP SEQUENCES IN BRAZILIAN HIV-1 AND NORTH AMERICAN/EUROPEAN SUBTYPE B VIRUSES

Sequence	Number (%) with the sequence	
	Brazil (n = 28)	Non-Brazilian, subtype B (n = 159) <sup>a</sup>
<b>Subtype B</b>		
H L G W G R A F	4 (14.0)	0
H M G W G R A F	4 (14.0)	3 (1.9)
H I G P G R A F	3 (11.0)	33 (21.0)
H M G L G R A F	2 (7.0)	0
H I G W G R A F	1 (3.6)	0
H M G W G R T F	1 (3.6)	2 (1.3)
H V G W G R A L	1 (3.6)	0
H I G P G R A W	1 (3.6)	0
S I G P G R A F	1 (3.6)	9 (5.7)
H M G P G R A Y	1 (3.6)	0
N I G P G R A F <sup>b</sup>	1 (3.6)	6 (3.8)
Q Y G T G G G A	1 (3.6)	0
H I G W G R A I	1 (3.6)	0
R I G P G S A F	1 (3.6)	0
P I A P G S A W	1 (3.6)	0
H I G F G R T L	1 (3.6)	0
H V G V G R A F	1 (3.6)	0
P L G P G R A F <sup>c</sup>	1 (3.6)	0
H L G P G G A F	1 (3.6)	0
P I G P G R A F	0	11 (7.0)
H I A P G R A F	0	6 (3.8)
T I G P G R A F	0	6 (3.8)
Other	0	83 (52.2)

<sup>a</sup> From Ref. 5.

<sup>b</sup> RJ I01, Putative subtype B/F recombinant.

<sup>c</sup> RJ I02, Subtype F.

studies will be necessary to evaluate the frequency of subtype F in Brazil, and the variation seen in the V3 region of this subtype. It should be noted that 5 of the 32 Brazilian samples analyzed here (15%) were not successfully amplified with 1 set of nested *env*-specific primers. The RJ IO3 subtype F sample was one of these five samples, and yielded amplified DNA only with another set of *env*-specific primers. Although insufficient DNA from the other four samples precluded further analysis, it is possible that they too contained F (or other divergent) subtype genomes.

The average nucleotide diversity in the V3 region within the Brazilian subtype B samples was 13.5%, which is similar to the 11.2% average observed among the non-Brazilian subtype B sequences shown in Fig. 2. This suggests that HIV-1 has been circulating in the Brazilian population for approximately the same period as in the United States.<sup>24</sup> This contrasts with the relatively recent spread of HIV-1 infection in Thailand<sup>35</sup> and India,<sup>36</sup> which has been characterized by a high degree of sequence conservation among the HIV-1 isolates from epidemiologically unrelated individuals.

Differences in the critical tetrapeptide "crown" of the V3 loop<sup>5,13-16</sup> are seen when the Brazilian subtype B sequences are compared to North American/European type B sequences. Whereas the GPGR tetrapeptide is present in 65% of North American/European samples so far analyzed,<sup>5</sup> it is present in only 28.5% of Brazilian sequences presented here. Eighty-seven percent of North American/European sequences contain proline (P) in the second position,<sup>5</sup> compared to 39% of the Brazilian sequences, whereas 43% have tryptophan (W) and 18% have other amino acids. High variability in the V3 crown tetrapeptide has also been found in non-B subtypes, where only 39% of isolates have proline at the second position.<sup>37</sup> However, a majority of non-B HIV-1 sequences (16 of 18 subtype A, C, D, and E sequences, compiled in Ref. 5) contain glutamine (Q) at the fourth position, whereas Brazilian (86% of sequences analyzed here) as well as North American/European sequences have

Also relevant is the previous study by Bongertz *et al.*,<sup>23</sup> which showed low reactivity of sera from HIV-1-infected Brazilians when tested against peptides corresponding to the region just downstream from GPGR in the V3 loop of the HIV-1 MN and SC isolates, described as predominant serotypes in North America and Europe.

In addition to showing important differences when compared to North American/European HIV-1 isolates, Brazilian sequences also show a high degree of variation in the V3 region. A frequent change of the IHI motif in the V3 loop to IHM or IHL was detected in the samples analyzed here, in addition to variability in regions flanking the V3 loop. These amino acid changes predict variation in glycosylation and net charge in this region.

Findings similar to those presented here have been published by Potts *et al.*,<sup>21</sup> including detection of one example of what is now referred to as subtype F,<sup>5,24,30</sup> with the interesting difference that most of their samples were obtained from the Bahia region of Brazil to the north of Rio de Janeiro and São Paulo.

The immunological impact of the significant differences observed here between Brazilian and North American/European isolates, and the variability seen among the Brazilian isolates in sequences important for neutralizing antibodies and cytotoxic T cells, should be taken into account for future immunoprophylactic programs to be established in Brazil.

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