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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 mV·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 mv·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

HUMAN IMMUNODEFICIENCY VIRUS type I (HIV-I) 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.[1-3] HIV-I *in vivo* has also been shown to consist of a population of related genomes that can change with time.[4] Serologically defined subtypes of HIV-1 have been reported,[2] and seven sequence-defined subtypes have been defined that differ by up to 35% in the surface (SU) coding portion of the *env* gene.[5] In contrast, the diversity within an individual usually ranges up to no more than 7%.[5,6] The importance of this genetic variation in terms of pathogenesis and vaccine development has been discussed by Wolfs *et al.*[7]

The highly variable V3 region of the envelope glycoprotein gp 120 is functionally important in that it (I) is a determinant for T cell and macrophage cellular tropism,[8-10] (2) is involved in determining syncytium induction phenotype and replication rate,[11,12] (3) contains the epitope recognized by antibodies that neutralize HIV infection *in vitro*, the so-called principal neutralizing determinant (PND),[13-16] and (4) stimulates a potent immune response by cytotoxic and helper T lymphocytes.[17-19] 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 AIOS cases, information is scarce with regard to the molecular diversity of Brazilian HIV-I strains,[20,21] 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-I 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.[22,23]

We have evaluated the degree of polymorphism among Brazilian HIV-I 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-I 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 AIOS clinic at the Adolpho Lutz Institute, were included in this study (Table I). Samples were obtained in 1990, 1991, and 1992. Ali patients were serologically positive as confirmed by enzyme-linked immunosorbent assay (ELISA),

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

Subject[a]	*Year*	*Risk group*[b]	*Sex*	*CDC clinical class*	*CD4+ cells (per mm³)*
RJ 12	1990	Heterosexual	F	Ii	444
RJ 14	1990	Bisexual	M	Ii	NA[c]
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[d]	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[d]	1991	Bisexual	M	II	503
RJ 379	1991	Heterosexual	F	II	657
RJ 477	1992	HeterosexulJ.l	M	II	981
RJ 478	1992	Heterosexual	F	II	401
RJ 483	1992	Heterosexllal	M	NA	496
RJ 484	1992	Heterosexual	F	NA	247
RJ 485[d]	1992	Heterosexual	F	II	494
RJ 491[d]	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 101	1992	Heterosexual	F	Ii	1031
RJ 102	1992	Heterosexual	F	II	NA
RJ 103	1992	Heterosexual	F	II	NA
RJ104	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

[a] RJ, Rio de Janeiro; SP, Sãn Paulo.
[b] Heterosexual females, sexual partners of HIV-seropositive individuais; heterosexual males, sexual intercourse with prostitlites. IVDU, Intravenous drug user.
[c] NA, Not available.
[d] Samples not amplified with *env* primer sets.

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

DNA preparation from peripheral blood mono nuclear cells

Genomic DNA was extracted by the proteinase K-sodium dodecyl sulfate (SDS), organk extraction method from 10^6 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 elhylenediaminetetraacetic acid (EDTA), containing 1% SDS and proteinase K (100 mgJml), incubated at 37°C ovemight, extracted with phenol (v:v), phenol-chloroforrn, and chloroforrn, and lhen 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-I 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.[24] In the first round, primers ED3 (5' TIAGGCATC-TCCfATGGCAGGAAGAAGCGG) (positions 5956-5986 of lhe HXB2 genome) and EDI2 (5' AGTGCTTCCfGCfGCfC-CCAAGAACCCAAG) (positions 7792-7822) were used. Aliquots (5 ml) of the first PCR round were reamplified using ES7 (5' TGTAAAACGACGGCCAGTCfGTI AAATGGCAGTCf AGC) (positions 7001-7021) and ES8 (5' CAGGAAACAGC-TA TGACCCACTICTCCAA TIGTCCCCACA) (positions 7646-7667) primers. The PCR conditions consisted of 3 cycles at 97°C (I min), 55°C (I min), and nOC (2 min), followed by 32 cycles at 95°C (I min), 55°C (I min), and nOC (2 min), with a IO-min extension at nOC in lhe last cycle. The buffer used was 10 mM Tris-HCI (pH 8.3), 50 mM KCI, a 200 mM concentration of each dNTP, and a 0.2 IJ.Mconcentration of each primer, and reactions contained 2.5 units of *Taq* polymerase (Perkin-Elmer, Norwalk, Cf) in a volume of 50 ml. In the first round, 1.25 mM $MgCl_2$ was used, and in the second 1.8 mM $MgCl_2$ was used.

Direct DNA sequencing of polymerase chain reaction product

To sequence directly the PCR product obtained above, a 5-ml aliquot of the double-stranded DNA product of the second-round PCR was converted to single-stranded DNA in an asymmetric PCR reaction[25] using one oligonucleotide primer (ES8). Asymmetric PCR conditions corresponded to 40 cycles at 95°C (I min), 55°C (I min), and nOC (2 min) with a IO-min extension at nOC in the last cycle in the same buffer as described above. After asymmetric PCR, the single-stranded DNA samples were purified with GeneClean (Bio 101, La 1011a,CA) and sequenced using the dideoxy chain terrnination method (Sequenase version 2.0; United States Biochemical, Cleveland, OH), with ES7 as sequencing primer.

Amplification and cloning of HIV-I DNA from peripheral blood mono nuclear cell DNA

DNA of sample RJ 103, which could not be amplified with the above set of *env* primers, was successfully amplified using ED3 (5' TI AGGCATCTCCT ATGGCAGGAAGAAGCGG, positions 5956-5985 of the HXB2 genome) and EDI4 *env* primers (5' TCTIGCCTGGAGCfGCTIGATGCCCCAGAC, 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 1-5' -2KSI and 1-3' -2-KSI-2 primers ofWolfs *et al.*?6 (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 $MgCl_2$. 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 pAMPI vector DNA (GIBCO-BRL), and transforrned into *Escherichia coli* DH5a 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-I PCR product without cloning yielded a consensus nucleotide sequence of the predominam V3 loop and flanking regions for ali samples except Rl 103. The Rl 103 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-l isolates,5 using the program MASE.[27] A phylogenetic tree was constructed using the PHYLIP 3.5c package of computer programs.[28] The branching order was deterrnined using DNADIST (maximum likelihood method) and FITCH (with global rearrangements). Bootstrap analysis was done using the programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE. Sequences described in this study have been assigned GenBank accession numbers UOO400-U00427.

RESULTS

Patient population

Epidemiological and laboratory data from patients enrolled in lhis study are presented in Table I. *env* gene sequences were successfully amplified from PBMCs of 28 of these 32 individuais (see the next section). Of lhe 28, at least 4 samples (RJ 10 I, Rl 102, RJ 103, and RJ 104) were obtained from women wilh documented recent seroconversion. Nineteen samples were obtained from asymptomatic HIV-I-seropositive individuais and 5 from patients with AIDS-related complex (ARC)/AIDS. Clínical data were not available for four patients. Eighteen (15 women and 3 men) of the 28 individuais analyzed attributed infection to heterosexual intercourse. No HIV-l-positive coupies were included in this study in order to obtain a broad spectrum of HIV-I diversity in Brazil, that is, ali samples are from presumed epidemiologically unrelated individuais.

Polymerase chain reaction of env *DNA from Brazilian patients*

A region ofthe viral envelope gene *(env)* consisting ofthe 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 individuais. 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,29 suggesting that viruses within these individuais might be divergent at primer annealing sites within *env*. Indeed, the *env* gene of one of these remaining sequences, sample RJ 103, could be PCR amplified using a different nested set of env-specific primers (see Materiais and Methods). Insufficient DNA was available from the other four samples (RJ33, RJ370, R1485, RJ491) 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. I. 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 10 I and RJ 103) in the region shown in Fig. I 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. I, enough sequence was obtained to determine that, with the exceptions of RI 10 I and RJ 103, ali 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 103 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 103 was only 8.3% divergent from HIV sequence 7944 described in Brazil[21] and 11.8 to 14.9% divergent from several characterized sequences from Romanian children[30] (Figs. I and 2). Therefore, RJ 103 and the unique 7944 sequence from Brazil described by Potts *et al.*[21] can be classified as members of the newly identified HIV-I envelope sequence subtype F.5.24.30-32 Interestingly, the differences between RJ 103 and other Brazil-

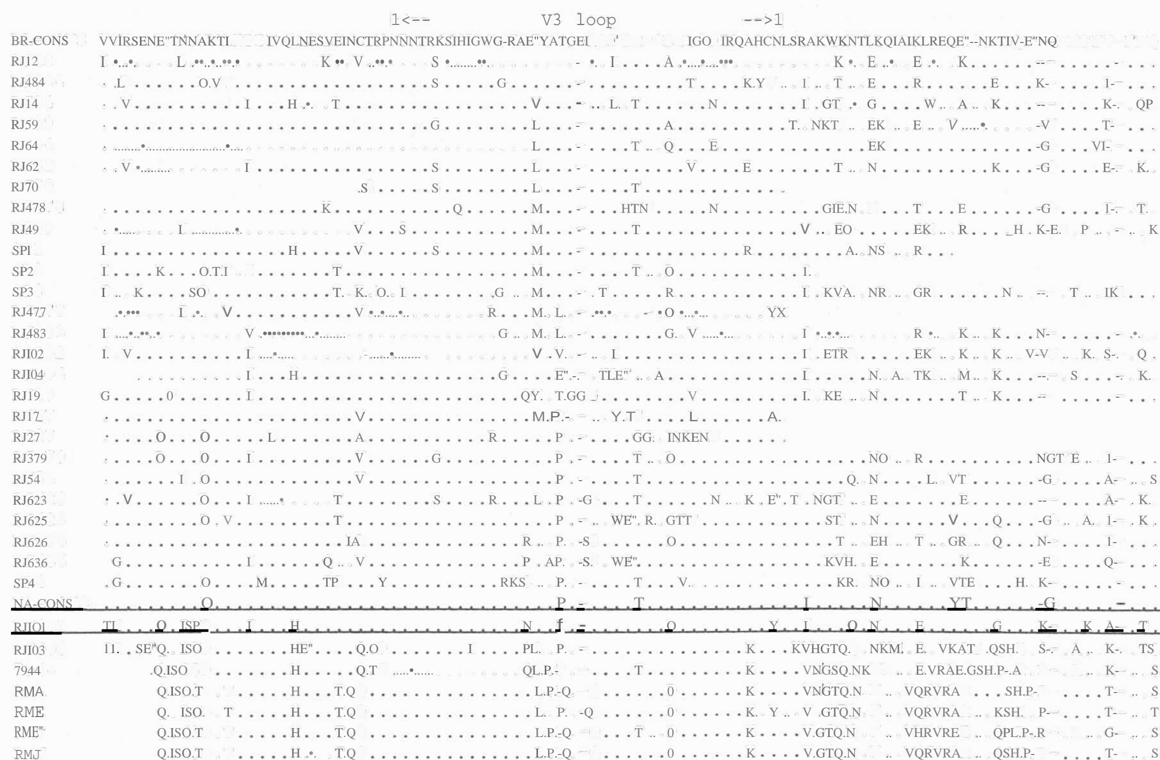

FIG.1. The alignment of Brazilian, B subgroup consensus HIV-l and F subgroup sequences in the V3 region. The putative recombinant sequence RJ 101 is shown between B subgroup sequences above it and F subgroup below. Consensus sequences were generated separately for all Brazilian and North American/European 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[30] and F subtype sequence 7944 from Brazil[21] (GenBank accession numbers L 19571, L 19575, L 19576, L 19579, and L 19237, respectively) are also shown.

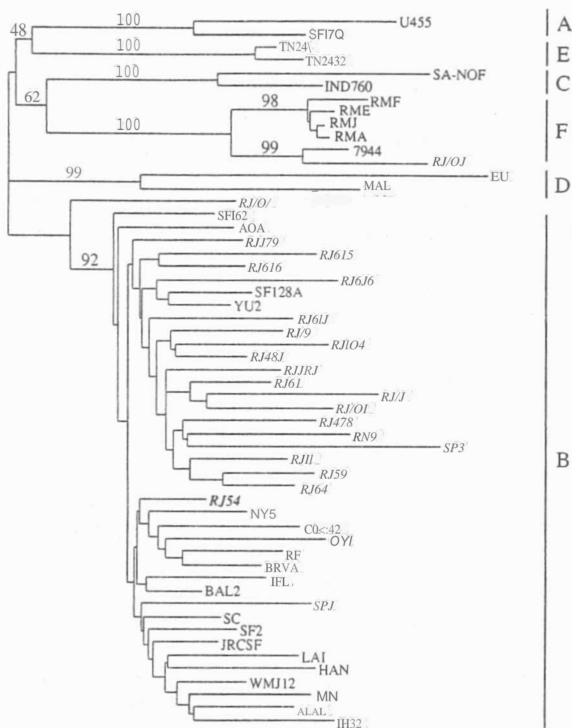

FIG.2. Phylogenetic tree for 22 Brazilian HIV-l sequences described in this article (shown in bold italics), 20 subtype B sequences, 5 subtype F sequences, and representative sequences from other HIV-l subtypes. The tree was generated for the fragment of the envelope gene shown in Fig. 1, excluding the first 15 nucleotides not sequenced in the Romanian samples.[30] Only completely sequenced Brazilian C2V3 sequences were included. Phylogenetic analysis was done using the PHYLIP programs[28] (see Materiais and Methods). The numbers near the major branches show how many times out of 100 bootstrap repetitions the sequenes to the right clustered together (putative recombinant virus RI 101 was excluded from bootstrap analysis). HIV-I isolates are from the following countries: all B subtype from the United States, except OYI (accession number M26727) from Gabon and HAN[S] from Germany; 7944 from Brazil; EU (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 10calized 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-I *env* sequences representing subtypes A-E[S] 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 RI Ia 1 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 (QJS.H in Fig. 1) and two silent mutations (not shown) present in the N-terminal region of RI Ia I and all of the subtype F sequences in Fig. I, which are either not present, or are rare, in the corresponding region of subtype B sequences. The potentially recombinational origin of RI 101 is also evident in Fig. 2, where it is positioned as an intermediate between B and other subtypes. Additional sequencing of RI Ia I and RI 103 is in progress to address the hypothesis that RI Ia I is a recombinant between B and F HIV-l subtypes. It is noteworthy that the one subtype F sequence and the putative B/F recombinant sequence (RI 103 and RI Ia I, respectively) were obtained from recent seroconverters.

Differenees between Brazilian and North Amerieanl European subtype B V3 loop sequenees

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.[S] The Brazilian subtype F sequences RI 103 and 7944[21] and the putative recombinant sequence RI Ia I all had the GPGR motif, whereas the Romanian subtype F sequences had GPGQ. The Romanian sequences also had other characteristic amino acids outside ofthis motif (Fig. I). 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-I samples was compared to 159 North American/European subtype B samples[S] in Table 2. Eighteen of28 Brazilian samples analyzed (64%) were unique to Brazil, including the octamer HLGWGRAF, which was present in 4 of 28 samples analyzed (14%). This demonstrates significant amino acid differences between Brazilian and North American HIV-I envelopes in this immunologically important epitope.

A comparison of the 35 amino acids corresponding to the V3 loop between Brazilian subtype B HIV-I samples and some prevalent North American/European HIV-l strains under evaluation for use in vaccine development showed that the levei 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 Americal/European consensus sequence and these various strains are on average higher (75,83,89, and 89%, respectively).

Sequeneing of tissue eulture 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[32] yielded similarities of 96 and 93% for sample pairs from subjects RI 623 and RI 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 viroses obtained after a single passage in PBMCs. Primary isolates recovered after cocultivation from nine Brazilian PBMC samples[33] will be useful for the evaluation of antigenic variability of Brazilian HIV-I strains in functional assays such as viral neutralization.

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

Sequence	Number (%) with the sequence	
	Brazil (n = 28)	Non-Brazilian, subtype B (n = 159t
Subtype B		
HL GWGRAF	4 (14.0)	0
HMGWGRAF	4 (14.0)	3 (1.9)
H I GP GRAF	3 (11.0)	33 (21.0)
HMGL GRAF	2 (7.0)	0
H I GWGRAF	I (3.6)	0
HMGWGRTF	I (3.6)	2 (1.3)
HVGWGRAL	I (3.6)	0
H I GP GRAW	I (3.6)	0
S I GP GRAF	I (3.6)	9 (5.7)
HMGP GRAY	I (3.6)	0
N I GP GRAF[b]	I (3.6)	6 (3.8)
QY GT GGGA	I (3.6)	0
H I GWGRAI	I (3.6)	0
R I GPGSAF	I (3.6)	0
P I AP GSAW	1 (3.6)	0
H I GF GRTL	1 (3.6)	0
HVGVGRAF	I (3.6)	0
P L GP GRAF"	I (3.6)	0
HL GP GGAF	I (3.6)	0
P I GP GRAF	0	Ii (7.0)
H I AP GRAF	0	6 (3.8)
T I GP GRAF	0	6 (3.8)
Other	0	83 (52.2)

a From Ref. 5.
b RJ 10I, Putative subtype B/F recombinant.
c RJ 102, Subtype F.

DISCUSSION

HIV-I strains with at least two envelope sequence subtypes, B and F, are cocirculating in Brazil, with a predominance of samples belonging to subtype B. Subtype F was identified in HIV-I samples from Romania.[30] In addition to the findings presented here, two other studies have detected subtype F sequences in Brazil.[24,31] A third report of 22 Brazilian sequences[21] detectd one unusual specimen (7944) that, although not categorized as F in the study, does appear to belong to this subtype as well. Only 1 (RJ 103) of 28 Brazilian HIV-I samples analyzed in this study belongs to the F subtype. This provirus shows divergence from subtype B mainly in the regions flanking the V3 loop, and has only three potential N-linked glycosylation sites in the region sequenced as compared to five to eight in the subtype B HIV-I samples and eight in the Romanian subtype F samples. This sample has also been placed into the F subtype, using PCR product encompassing the V1 through V5 regions of *env* in the heteroduplex mobility assay,[24] as well as by more complete sequencing.[34] The latter studies confirm the extensive difference between the RJ 103 sequence and subtype B over a longer stretch of DNA than presented here. Despite these differences, the V3 loop region of these first few subtype F sequences are similar to the B-subtype Brazilian strains. Further 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 103 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-I has been circulating in the Brazilian population for approximately the same period as in the United States.[24] This contrasts with the relatively recent spread of HIV-I infection in Thailand[35] and India,[36] which has been characterized by a high degree of sequence conservation among the HIV-I isolates from epidemiologically unrelated individuais.

Differences in the critical tetrapeptide "crown" of the V3 loop[5,13-16] 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,[5] 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,[5] 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.[37] However, a majority of non-B HIV-I 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 conserved arginine (R) at this position. Furthermore, the average divergences within two major groups of Brazilian subtype B sequences, distinguished by the presence of W or P within the crown, are similar (13.4 versus 12.9%, respectively). The P-W substitution requires three nucleotide changes (CCA-TGG), and is extremely rare among North American/European sequences, suggesting that Brazilian sequences with W in the V3 loop have a common phylogenetic ancestor.

The GWGR sequence in the tip of the V3 loop is rare in ali HIV-I subtyps so far analyzed, but has been identified in Brazil (data presented here as well as in Ref. 21), and in 3 of 29 isolates from Japan, I of which was obtained from a heterosexual woman who had emigrated from South America.[38] The high frequency of the GWGR pattem observed in this study is unlikely to be due to a bias related to the type of HIV-I transmission, because it is found in heterosexual men and women as well as homosexual and bisexual men.

The amino acid differences in the tip of the V3 loop detected in this study can lead to important changes in antigenicity, as demonstrated by Gomy *et ai.*,[39] who showed that the binding of a human monoclonal antibody with high neutralization activity to an HIGPGR hexapeptide was completely abrogated when amino acid changes were introduced at the third, fourth, and sixth residues, or when proline was replaced by tryptophan.

Also relevant is the previous study by Bongertz *et al.*, 23 which showed low reactivity of sera from HIV-I-infected Brazilians when tested against peptides corresponding to the region just downstream from GPGR in the V3 loop of the HIV-I 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-I 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.*,21 including detection of one example of what is now referred to as subtype F,5.24.30 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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REFERENCES

1. Asjö B, Albert J, Karlsson A, Morfelt-Månson, Biberfeld G, Lidman K, and Fenyö EM: Replicative properties of human immunodeficiency virus from patients with varying severity of HIV infection. Lancet 1986;ii:660-662.
2. Cheng-Mayer C, Homsy J, Evans LA, and Levy JA: Identification of human immunodeficiency virus subtypes with distinct patterns of sensitivity to serum neutralization. Proc Natl Acad Sci USA 1988;85:2815-2819.
3. Tersmetle M, Gruters RA, de WolfF, de Goede REY, Lange JMA, Schellekens PIA, Goudsmit J, Huisman HG, and Miedema F: Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of acquired immunodeficiency syndrome: Studies on sequential HIV isolates. J Virol 1989;63:2118-2125.
4. Simmonds PP, Zhang LQ, McOmish F, Balfe P, Ludlam CA, and Brown AJL: Discontinuous sequence change of human immunodeficiency virus (HIV) type 1 *env* sequences in plasma viral and lymphocyte-associated proviral populations in vivo: Implications for models of HIV pathogenesis. J Virol 1991;65:6266-6276.
5. Myers G, Korber B, Wain-Hobson S, Smith RF, and Pavlakis GN (Eds.): *Human Retroviruses and AIDS* 1993: A *Compilation and Analysis of Nucleic Acid and Amino Acid Sequences.* Los Alamos National Laboratory, Los Alamos, New Mexico, 1993.
6. Kusumi K, Conway B, Cunningham S, Berson A, Evans C, Iversen AKN, Colvin D, Gallo MV, Coutre S, Shpaer EG, Faulkner DV, deRonde A, Volkman S, Williams C, Hirsch MS, and Mullins JI: Human immunodeficiency virus type I envelope gene structure and diversity in vivo and after co-cultivation in vitro. J Virol 1992;66:875-885.
7. Wolfs TF, Nara PL, and Goudsmit J: Genotyping and phenotypic variation of HIV-I: Impact on AIDS pathogenesis and vaccination. Chem Immunol 1993;56: 1-33.
8. Shioda T, Levy JA, and Cheng-Mayer C: Macrophage and T-cell line tropisms of HIV-I are determined by specific regions of the envelope gp120. Nature (London) 1991;349: 167-169.
9. Takeuchi Y, Akutsu M, Murayama K, Shimizu N, and Hoshino H: Host range mutant of human immunodeficiency virus type I; modification of cell tropism by a single point mutation at the neutralization epitope in the *env* gene. J Virol 1991;65: 1710-1718.
10. Hwang SS, Boyle TJ, Kim-Lyerly H, and Cullen BR: Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. Science 1991;253:71-74.
11. Fouchier RAM, Groenink M, Kootstra NA, Tersmette M, Huisman HG, Miedema F, and Schuitemaker H: Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp120 molecule. J Virol 1992;66:3183-3187.
12. Kuiken CL, de Jong J-J, Keulen W, Tersmette M, and Goudsmit J: Evolution of the V3 envelope domain in proviral sequences and isolates of human immunodeficiency virus type 1 during the transition of the viral biological phenotype. J Virol 1992;66:4622-4627.
13. Palker TJ, Clark ME, Langlois AJ, Matthews TJ, Weinhold KJ, Randall RR, Bolognesi DP, and Haynes BF: Type-specific neutralization of the human immunodeficiency virus with antibodies to env-encoded synthetic peptides. Proc Natl Acad Sci USA 1988;85: 1932-1936.
14. Javaherian K, Langlois AJ, McDanal C, Ross KL, Eckler LI, Jellis CL, Profy AT, Rusche JR, Bolognesi DP, Putney SD, and Matthews TJ: Principal neutralizing domain of the human immunodeficiency virus type envelope protein. Proc Natl Acad Sci USA 1989;86:6768-6772.
15. Goudsmit J, Debouck C, Meloen RH, Smit L, Bakker M, Asher DM, Wolff AV, Gibbs CJJ, and Gajdusek C: Human immunodeficiency virus type 1 neutralization epitope with conserved architecture elicits early type-specific antibodies in experimentally infected chimpanzees. Proc Natl Acad Sci USA 1988;85:4478-4482.
16. LaRosa GJ, Davide JP, Weinhold K, Waterbury K, Profy AT, Lewis JA, Langlois AJ, Dreesman GR, Boswell RN, Shadduck P, Holley LH, Karplus M, Bolognesi DP, Matthews TJ, Emini EA, and Putney SD: Conserved sequence and structural elements in the HIV-1 principal neutralizing determinant. Science 1990;249:932-935.
17. Takahashi H, Cohen J, Hosmalin A, Cease KB, Houghton R, Cornette JL, DeLisi C, Moss B, Germain RN, and Berzofsky JA: An immunodominant epitope of the human immunodeficiency virus envelope glycoprotein gp160 recognized by class I major histocompatibility complex molecule-restricted murine cytotoxic T lymphocytes. Proc Natl Acad Sci USA 1988;85:3105-3109.
18. Takahashi H, Nakagawa Y, Pendleton CD, Houghton RA, Yokomuro K, Germain RN, and Berzofsky JA: Induction of broadly cross-reactive cytotoxic T cells recognizing an HIV-1 envelope determinant. Science 1992;355:333-336.
19. Palker TJ, Matthews TJ, Langlois A, Tanner ME, Martin ME, Scearce RM, Kim JE, Berzofsky JA, Bolognesi DP, and Haynes BF: Polyvalent human immunodeficiency virus synthetic immunogen comprised of envelope gp120 T helper cell sites and B cell neutralization epitopes. J Immunol 1989;142:3612-3619.
20. Coulo-Fernandez J-C, Morgado MG, Ivo-dos-Santos J, and Galvao-Castro B: Molecular and biological diversity of HIV-1 in Brazil. Mem Inst Oswaldo Cruz 1992;87:249-255.

21. Potts K, Kalish M, LotlT, OrloffG, Luo C-C, Bemard M-A, Alves C, Badaro R, Suleiman J, Ferreira O, Schochelman G, Johnson W Jr, Ou C-V, and HoJ: Genelic helerogeneity ofthe V3 region oflhe HIV-I envelope glycoprotein in Brazil. AIOS 1993;7:1191-1197.
22. Carrow EW, Vujcic LK, Glass WL, Seamon KB, Raslogi SC, Hendry RM, Boulos R, Nzila N, and Quinnan GV Jr: High prevalence of antibodies 10 lhe gpl20 V3 region principal neUlra!-izing delerrninant of HIV -I MN in sera from Africa and lhe Americaso AIOS Res Hum Relroviruses 1991;7:831-838.
23. Bongertz V, Jonsson M, Rudby P, Morgado MG, Galvão-Caslro B, and Wigzell H: Reactivity of Brazilian sera wilh gpl20 V3 peptides. Brazilian J Med Biol Res 1994:27.
24. Oelwart EL, Shpaer EG, Louwagie J, McCutchan FE, Grez M, Rübsamen-Waigmann H, and Mullins JI: Genetic relationships delerrnined by a ONA heteroduplex mobility assay: Analysis of HIV-I *env* genes. Science 1993;262:1257-1261.
25. Burger H, Weiser B, Raherty K, Gulla J, Nguyen P-N, and Gibbs RA: Evolution ofhuman immunodeficiency virus lype I nucleotide sequence diversily among close conlacls. Proc Nat! Acad Sci USA 1991;88:11236-11240.
26. Wolfs TFW, Zwart G, Bakker M, and Goudsmil J: HIV-I genomic RNA diversification following sexual and parenteral virus lransmission. Virology 1992;189:103-110.
27. Faulkner OV and Jurka J: Multiple aligned sequence edilor (MASE). Trends Biochem Sci 1988;13:321-322.
28. Felsenslein J: PHYLIP-Phylogeny Inference Package. Cladistics 1989;5:164-166.
29. Sabino E, Cheng-MayerC, and Mayer A: An individual Wilh a high prevalence of a *tGt-defective* provirus in peripheral blood. AIOS Res Hum Relroviruses 1993;9: 1265-1268.
30. Oumilrescu O, Kalish ML, Kliks SC, Bandea CI, and Levy JA: Characterization of HIV -I strains isolaled from children in Romania: Identification of a new envelope sublype. J Infecl Ois 1994; 369:283-288.
31. Louwagie J, Oelwart EL, Mullins JI, McCulchan FE, Eddy G, and Burke OS: Genelic analysis of HIV-I isolales from Brazil reveals presence of lwo distinct genolypes. AIOS Res Human Relroviruses 1994;10:561-567.
32. Gallo O, Kimplon JS, and Oailey PJ: Comparalive sludies on use of fresh and frozen peripheral blood Iymphocyle specimens for isolation of human immunodeficiency virus and effecls of cell Iysis on isolalion efficiency. J Clin Microbiol 1987;25: 1291-1294.
33. Hendry RM, Viral and Rickeltsial Oiseases Laboralory, Califomia Public Health Foundation (Berkeley, CAl: Unpublished resulls, 1993.
34. Sabino EC, Shpaer EG, Morgado MG, Korber BTM, Oiaz R, Bongertz V, Cavalcanle S. Galvão-Castro B, Mullins n, and Mayer A: Idenlificalion of HIV-I proviral genomes recombinant between subtypes B and F in PBMC obtained from lwo individuals in Brazil. Manuscripl in preparation, 1993.
35. McCutchan FE, Hegerich PA, Brennan TP, Phanuphak P, Singharaj P, Jugsudee A. Berrnan PW, Gray AM, Fowler AK, and Burke OS: Genetic varianls of HIV -I in Thailand. AIOS Res Hum Relroviruses 1992;8: 1887-1895.
36. Oielrich U, Grez M, von Briesen H, Panhans B, Geil3endórfer M, Kühnel H, Maniar J, Mahambre G, Becker WB, Becker MLB, and Rübsamen-Waigmann H: HIV-I slrains from India are highly divergenl from prololypic African and US/European slrains, bul are linked to a Soulh African isolale. AIOS 1993;7:23-27.
37. Korber B, Wolinsky S, Haynes B, Kunslman K, Levy R, Furtado M, alto P, and Myers G: HIV-I inlrapalienl sequence diversity in lhe immunogenic V3 region. AIOS Res Hum Relroviruses 1992;8: 1461-1465.
38. Hallori T, Shiozaki K, Eda Y, Tokiyoshi S, Matsushila S, Inaba H, Fujimaki M, Meguro T, Yamada K, Honda M, Nishikawa K, and Takatsuki K: Characteristics of lhe principal neulralizing deterrninant of HIV-I prevalent in Japan. AIOS Res Hum Relroviruses 1991;7:825-830.
39. Gomy MK, Conley AJ, Karwoska S, Buschbinder A, Xu J-Y, Emini EA, Koenig S, and Zolla-Pazner S: Neutralization of diverse human immunodeficiency virus type I variants by an anti- V3 human monoclonal antibody. J Virol 1982;66:7538-7542.

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