RESEARCH NOTE

A Nationwide Effort to Sistematically Monitor HIV-1 Diversity in Brazil: Preliminary Results

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The Human Immunodeficiency Virus Type 1 (HIV-1) presents a high level of genetic variation leading to isolates with divergent nucleotide and amino acid sequences and distinct biological properties. This viral diversity is one of the main obstacles for the development of a universal effective vaccine. Based on HIV-1 *env* and *gag* gene sequence data, at least nine nearly equidistant genetic subtypes belonging to the M (major) group could be identified. They are designated from A through I and occurring in different geographic regions of the world (G Meyers 1994 *AIDS Res Hum Retroviruses 10*: 1317-1324, G Kostrikis et al. 1995 *J Virol 69*: 6122-6130). In addition, divergent HIV-1 viruses have been identified, and as could not be classified in any of these subtypes, were recently designated as group O (outlier) (M Van den Haesevelde et al. 1994 *J Virol 68*: 1586-1596, LG Gurtler et al. 1994 *J Virol 68*: 1581-1585, W Janssens et al. 1994 *AIDS 10:* 877-879).

Thus, to establish a surveillance program to monitor HIV-1 diversity in sites where HIV-1 candidate vaccine will be evaluated is of paramount importance for the selection of appropriate vaccine efficacy trials. In this context, the World Health Organization Global Programme on AIDS (WHO-GPA) organized a WHO Network for HIV-1 Isolation and Characterization, providing the basis of global mechanisms for monitoring HIV-1 variability (WHO Network for HIV-1 Isolation and Characterization 1994 *AIDS Res Hum Retroviruses 10:* 1327-1343).

Brazil is one of the vaccine trial sites selected by the WHO-GPA, together with Rwanda, Thailand and Uganda. So far, at least three different subtypes have been found in Brazil: B, F, and C. In fact, through an evaluation of 235 Brazilian isolates it was observed that subtype B was predominant (88.5%) and that only 8.9% and 1.7% of the samples were subtypes F and C, respectively (K Potts et al. 1993 *AIDS 7:* 1191-1197, J Louwagie et al. 1993 *AIDS 7:* 769-780, MG Morgado et al. 1994 *AIDS Res Hum Retroviruses 10:* 569-576, JC Couto-Fernandez et al. 1994 *AIDS Res Hum Retroviruses 10:* 1157-1163, WHO Network for HIV-1 Isolated and Characterization, 1994 *AIDs Rev Hum Retrov 10:* 1327-1343, B Galvão-Castro et al. 1995 *Actualizaciones en SIDA 3:* 173-178, E Sabino et al. 1995 2nd National Conference on Human Retroviruses and Related Infections, Washington, DC- USA, M Guimarães et al. 1995 1º Simpósio Brasileiro de Pesquisa Básica em HIV/AIDS abstract no. 17, RJ, Brazil). Interestingly, two samples (0.9%) showed to be variants resulting from a recombination between subtypes B and F (E Sabino et al. 1994 *J Virol 68:* 3640-6346, B Hahn et al. 1996 *J Virol* in press).

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Low levels of amino acid sequence conservation in the V3 loop were also seen between the Brazilian sequences and the HIV-1 prototypes currently in use for vaccine development. Indeed, the comparison with sequences of prevalent North American/European HIV-1 strains showed that the Brazilian subtype B sequences present amino acid replacements in some positions giving distinctive tetrameres at the tip of the V3 loop. In fact, the GWGR motif at the crown of the V3 loop was detected in 28 out of 71 (39.4%) isolates analyzed, while the highly conserved North American/European GPGR motif was observed in 25 samples (35.2%). Also, novel sequences were detected in 18 samples (25.4%) (MG Morgado 1994 in Processo de desenvolvimento de vacinas anti HIV/AIDS. Problemas e benefícios PN-DST/AIDS, Brazilian Ministry of Health). Moreover, the sequencing of the whole gp120 DNA of one Brazilian isolate with GWGR motif at the tip of the V3 loop showed an 89.1% homology of nucleic acid sequence with the prototype HIV-1 B subtype. The highest divergence was found in the V1-V3 regions (SM Costa et al. 1995 *Aids Res Hum Retroviruses 11*: 1243-1245).

In addition, Brazilians infected with HIV-1 presented a lower specific antibody response against V3 loop peptides of predominant prototypes of HIV-1 circulating in North America and Western Europe (EW Carrow et al. 1991 *AIDS Res Hum Retroviruses 7*: 831-838, V Bongertz et al. 1994 *Braz J Med Biol Res 27*: 1225-1236).

These results suggest that Brazilian HIV-1 strains have genetic and antigenic differences in comparison with North American/European prototype strains, which may hamper the success of immunoprophylatic programmes based on HIV-1 vaccine candidates currently proposed to be tested in Brazil.

Therefore a Brazilian Network for HIV-1 Isolation and Characterization (BNHIC) was established in March 1993, as part of the National Programme of HIV/AIDS Vaccine Development and Evaluation, nested with Sexual Transmitted Disease/AIDS Programme of the Brazilian Ministry of Health. This network has similar organizational structure to the WHO (*loc. cit.*). Briefly, BNHIC was organized on a three-tier basis inclunding primary site, central reference laboratory and secondary laboratories.

Initially the primary cities comprised three previously selected ones for HIV-1/AIDS vaccine evaluation located in the cities of Belo Horizonte, MG; Rio de Janeiro, RJ, and São Paulo, SP. These sites are responsible for the selection of volunteers, collection of blood specimens and the shipment of the blood samples to the Central Reference Laboratory.

The Central Reference Laboratory is the Advanced Laboratory of Public Health (LASP), Gonçalo Moniz Research Center, FIOCRUZ, in Salvador, BA. This laboratory is responsible for HIV-1 isolation, expansion and distribution of biological samples and reagents to the secondary laboratories. The Central Laboratory is also the HIV isolates national repository and is also responsible for transferring of technology and traning.

The biological, immunological and genetic characterization of the specimens is undertaken at the secondary laboratories: (1) AIDS and Molecular Immunology Laboratory, Department of Immunology, Oswaldo Cruz Institute, FIOCRUZ; (2) Infectious Disease Service, Department of Preventive Medicine, Federal University of Rio de Janeiro; (3) Microbiology and Immunology Laboratory, Adolfo Lutz Institute; (4) Molecular Virology Laboratory, Dept. of Genetics, Federal University of Rio de Janeiro; (5) Retrovirology Laboratory, Adolfo Lutz Institute; (6) Retrovirology Laboratory, Departament of Virology, Oswaldo Cruz Institute, FIOCRUZ; (7) Virus Laboratory, Basic Science Institute, Federal University of Minas Gerais.

The main objectives of the Brazilian National Network are (1) to develop a system for continuous monitoring the genetic and antigenic variability of HIV-1 isolates from different geographic regions of Brazil; (2) to generate basic information of genetic and antigenic properties of epidemiologically relevant HIV-1 strains that will enable the selection of antigenically appropriate candidate vaccines to be evaluated and potentially used in Brazil; (3) to participate in other international HIV-1 characterization efforts as part of the WHO Network for HIV Isolation and Characterization, or on a bilateral basis of collaboration with individual international research programs. This approach, using rigorous standardized research protocols, will enable a wider ranging analysis of HIV-1 samples from different regions of Brazil.

In order to achieve these goals, we have been carrying out a pilot study in one of three previously selected sites, (Rio de Janeiro, RJ) for future HIV vaccine evaluation.

Initially, 16 seropositive individuals were selected for this study. HIV-1 isolation and expansion were performed according to the standard procedures described elsewhere (WHO Guidelines for Standard HIV Isolation Procedures, Geneva, 1994). Briefly, PBMC of seropositive and seronegative individuals were separated in gradient of Ficoll Hypaque, from whole blood collected using EDTA. 8×10^6 PBMC from seronegative donors, previously stimulated with phytohemaglutinin, were cocultured with 2×10^6 patient cells in RPMI medium containing glutamine, penicilin, streptomy-

cin and 10% fetal calf serum in the presence of 5U/ml Interleukin-2. The co-cultures were incubated at 37ºC, 5% CO^2, up to 24 days. Culture medium was changed each three to four days and fresh donor cells were added on the 7th and on the 14th days. Co-cultures were monitored for the presence of the p24 antigen each three to four days and the positive supernatants were saved as virus stock.

HIV-1 isolates were biologically characterized using the methodology described elsewhere (WHO *loc. cit.*). Briefly, positive supernatants from primary cultures were used as the virus source to infect MT-2 cell lines. These cultures were monitored twice a week for p24 antigen and daily for syncitium formation. The isolates were also analyzed by heteroduplex mobility assay (HMA) in order to determine the subtypes of HIV-1 (E Delwart et al. 1993 *Science 262*: 1257-1261). The HIV-1 neutralization assay was performed following the techniques described previously (J Albert et al. 1993 *AIDS Res Hum Retroviruses 9:* 501-506).

The age, sex, presumed mode of transmission, clinical status of the patients, as well as results of HIV-1 isolation are shown in Tables I and II. The majority of the patients were males (75%). The age ranged from 15 to 61 years. Concerning the possible transmission route, 50% were male homossexual and/or bissexual, 43% were heterossexual, and in 6.25% the mode of transmission was undeterminated. We isolated HIV-1 in 12 out of 16 (75%) samples. The p24 antigen was always detected within 12 days of co-culture. Preliminary results of biologic characterization show that seven isolates induced syncytium formation in primary co-culture (PBMC) but only three of them induced syncytium in MT-2 cell line (Table II). Only one isolate induced syncytium in MT-2 cell line alone. The rate of virus isolation and the frequency of syncytium inducing (SI) isolates were higher than that observed in a previous study (WHO *loc. cit.*), which could be due to the variation of the clinical status of our patients. The genetic analyses of 11 samples using HMA revealed that 10 were B and 1 was F subtypes confirming previous studies.

Neutralization assays, carried out according to standard techniques in pre-activated peripheral blood mononuclear cells (PBMC) (Albert et al. *loc. cit.*) indicate that two of seven isolates tested were neutralized to at least 75% by their autologous plasma (28.6%) and a third isolate was neutralized to 50%. Six of the seven isolates (85.7%) were neutralized by at least one heterologous plasma from Brazilian HIV-1 infected individuals. Of 11 plasma evaluated as to their potency in neutralizing heterologous primary Brazilian HIV-1 isolates, nine (82%) were able to neutralize 75% of at least one isolate. All isolates tested (7/7 = 100%) were susceptible to > 75% neutralization by a pool of plasma from the patients envolved in this study. Of 12 plasma tested, nine (75%) were able to neutralize the reference isolate HIV-1 MN. These preliminary results indicate that one of the isolates (RJ95006) appears to be quite resistent to neutralization (autologous and heterologous), while another isolate (RJ95005) appears to be highly susceptible to neutralization, with all other isolates showing intermediate susceptibility. In summary, neutralization of Brazilian primary HIV-1 isolates appears to be similar to neutralization of other primary HIV-1 isolates described in the United States (PD Souza et al. 1995 *AIDS 9*: 867-874, JP Moore et al. 1995 *J Virol 69*: 122-130) and Europe (Eva-Maria Fenÿo 1994 personal communication and *9ème col Cent gardes* 103-107).

Some of the individuals enrolled in this pilot study did not fullfill the inclusion criteria required to participate in this research project. Most of them were infected for more than two years, including two cases of AIDS with CD4 cell counts below 200 cells/mm^3. In addition, some of them were under antiviral treatment. Thus, it is important to point out that the recruitment of individuals not strictly meeting the inclusion criteria in this study, such as: (a) be a recent seroconverted or to have less than two years of infection; (b) have CD4 cell counts above 200 cells/mm^3 and (c) not be under

TABLE I

Epidemiological and clinical data and CD_4^+ cell counts from individuals from Rio de Janeiro, RJ, Brazil

Patients	Sex	Age	PTR[a]	Clinical status	CD4/ mm^3
RJ001-95	M	35	homo	asympt	670
RJ002-95	M	28	bi	AIDS	519
RJ003-95	M	15	homo	asympt	379
RJ004-95	M	61	?	AIDS	<50
RJ005-95	M	58	homo	AIDS	191
RJ006-95	M	26	hetero	asympt	ND
RJ007-95	F	30	hetero	asympt	673
RJ008-95	F	42	hetero	asympt	473
RJ009-95	F	43	hetero	asympt	ND
RJ010-95	M	37	hetero	asympt	612
RJ011-95	M	49	bi	asympt	1098
RJ012-95	M	28	homo	asympt	330
RJ013-95	M	45	hetero	asympt	1191
RJ014-95	M	53	bi	asympt	115
RJ015-95	M	32	bi	asympt	549
RJ016-95	F	48	hetero	asympt	689

a: presumed transmission route (homo= male homosexual; bi= male bisexual; hetero= heterosexual)

TABLE II

Genetic and phenotipic characterization of HIV-1 isolates from individuals from Rio de Janeiro, RJ, Brazil

					Neutralization			
	HIV	CPE[a]		TCID[b]	Susceptibility[c]		Potency[d]	Subtypes
Patients	isolation	PBMC	MT-2	50%	aut	het	het	HMA[e]
RJ001-95	Y	+	+	<2	NA	NA	+	B
RJ002-95	Y	+	-	50	+	+	+	B
RJ003-95	Y	+	-	<2	NA	NA	+	F
RJ004-95	Y	+	-	750	-	+	+	B
RJ005-95	Y	-	-	750	+	+	+	B
RJ006-95	Y	-	+	250	-	-	+	B
RJ007-95	N	-	-	NA[f]	NA	NA	ND	B
RJ008-95	N	-	-	NA	NA	NA	ND	B
RJ009-95	Y	+	-	30	-	+	-	B
RJ010-95	Y	+	+	50	-	+	-	B
RJ011-95	Y	-	-	10	NA	NA	+	B
RJ012-95	Y	+	+	ND[g]	ND	ND	ND	ND
RJ013-95	N	-	-	ND	ND	ND	ND	ND
RJ014-95	Y	-	-	ND	ND	ND	ND	ND
RJ015-95	Y	-	-	ND	ND	ND	ND	ND
RJ016-95	N	-	-	ND	ND	ND	ND	ND

a: cytopathic effect (in PHA activated peripheral blood mononuclear cells and in MT-2 cells), as measured by the presence or not of multinucleated giant cells (syncitia)
b: TCID = tissue culture infectious dose 50%
c: susceptibility of primary HIV-1 isolates to neutralization by autologous (aut) and heterologous (het) sera from Brazilian HIV-1 infected individuals.
d: potency of the sera from these individuals in neutralizing heterologous (het) primary Brazilian isolates.
e: heteroduplex mobility assay
f: not available
g: not done

antiviral treatment, could jeopardize the information required for future vaccine efficacy trials.

Nevertheless, this pilot study has demonstrated the feasibility of the BNHIC, as well as the possibility of the HMA usage for large scale molecular epidemiological studies in Brazil. This will enable us to establish a sentinel surveillance on the prevalence and the dynamic of different HIV-1 genetic subtypes in various population groups in Brazil.

Finally, we should emphasize that a well organized and integrated national effort is of paramount importance for establishing a successful surveillance system to monitor HIV-1 diversity on a nationwide scale.

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