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BIOLOGICAL CHARACTERIZATION OF A NEW SUBTYPE-A VARIANT OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 FROM NIGERIA

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ABSTRACT: We have isolated a new variant of HIV-1 from Nigeria. The virus was recovered from the peripheral blood mononuclear cells (PBMCs) of an apparently asymptomatic, 23-year old male from Ibadan, Nigeria. The *in vitro* host range indicates that this virus is highly cytopathic to normal PBMCs, established T-cell lines and the monocytic cell line U937. The highest virus titre was obtained in freshly isolated macrophage/monocyte cells which also showed the least cytopathology. Based on the DNA sequence analysis of cDNA clones containing the *env* gene, this virus has been classified as HIV-subtype or clade A. None of the five viruses currently assigned to clade A have been isolated from Nigeria. The clade A viruses also have not been shown to exhibit extreme cytopathicity of lymphocytes and macrophage tropism observed in the Nigerian strain. Since most macrophage-tropic viruses have been associated with "neurotropism", the isolation of an HIV-1 strain from the blood of an individual with no known neurological disorders indicates that this unique combination of cell-tropism may be essential for the rapid onset of HIV disease. This is the first report of an HIV-1 isolate from Nigeria.

Key Words: AIDS; HIV-1; Clade A; Macrophage-tropic viruses; Neurotropism

INTRODUCTION

The Acquired Immunodeficiency Syndrome (AIDS) is fast becoming a pandemic problem as the number of cases reported annually from all parts of the world is increasing steadily (1). Although AIDS is a global problem, the human population in sub-Saharan Africa has been disproportionately affected with a high incidence of Human Immunodeficiency Virus (HIV) infection (2). As of June, 1993, 247,577 out of 718,894 cases of AIDS reported to the World Health Organization were from Africa (1).

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The first report of HIV infection in Nigeria was published in 1986 (3). Since then, only limited reports of HIV epidemiology or AIDS in this country have been published (4). Although genetic or phenotypic variability and differences in the biological properties of HIV isolates have been shown to be major factors influencing the course of HIV infection (5-7), it is still not clear which HIV strains are pathogenic and why. Furthermore, predominant sub-type(s) of HIV in different geographical regions of Africa have not been identified. The limited data available on the biological, immunological and molecular properties of HIV isolates from Africa are based on studies performed on only a few HIV-1 and HIV-2 strains (8). This information would be necessary for the development of an effective vaccine

for an early intervention and/or prevention of HIV disease.

Our recent study (9) on several thousand sera collected from six categories of people living in 21 locations in Nigeria from 1985 to 1990 showed more than ten-fold increase in the prevalence rate of antibody to HIV-1 and HIV-2 between 1986 and 1990. We have further identified antibody reactivities to two or more distinct retroviruses in the same serum (10). However, isolation of HIV-1 or HIV-2 from Nigeria has not been reported. We present here the virologic and biologic properties of the first HIV strain isolated from a male blood donor in Nigeria.

Although the subject was asymptomatic at the time of blood donation, this virus may be associated with the clinical expression of the disease as this individual died within a few months after donating the blood from which the HIV-1 was isolated.

MATERIALS AND METHODS

A 23-year old male, auto-mechanic donated one pint of blood in November, 1991, for a relation who required a blood transfusion in a hospital in Ibadan, Nigeria. At the time of blood donation, the subject did not have any AIDS-related symptoms and appeared healthy (Table 1).

As part of the routine transfusion safety procedure, an aliquot of plasma from the subject's blood, collected in acid-citrate-dextrose (ACD) anticoagulant, was tested for antibodies to HIV-1 and HIV-2 using the Wellcozyme Enzyme Immunoassay (EIA) according to the manufacturer's instructions. Within 24 hours of collection, peripheral blood mononuclear cells (PBMCs) were isolated from 30 ml of whole blood by Ficoll-Hypaque density gradient centrifugation. Isolated PBMCs were washed 3 times in phosphate buffered saline (PBS) and frozen at -70^{o}C in aliquots of approximately 4 x 10^{6} cells/2ml of freezing medium containing 80% inactivated foetal bovine serum, 10% RPMI-1640 and 10% DMSO. Frozen cells and plasma were transported on dry ice from the Department of Virology, University College Hospital, Ibadan, Nigeria, to the Laboratory of Viral Oncology and AIDS Research, University of Southern California (USC), CA, USA, and further stored for several months at -85oC until analyzed.

The plasma was retested at USC for antibodies to HIV-1, HIV-2, HTLV-I and HTLV-II using EIA by Organon Teknika Corporation, NC and Genetic Systems Corporation, Seattle, WA, USA. Samples consistently reactive in EIA were confirmed for the pattern of antibody reactions bt Western blots.

Table 1: Source of HIV-1_{IbNg}.

23 year old male
Blood donor
Asymptomatic at the time of blood donation
HIV-seropositive
Plasma HIV-1 p24 antigen positive
HIV-2 seronegative
HTLV-I seronegative
HTLV-II seronegative
Developed symptomatic illness 6 months after blood donation and died 4 months later

Virus Isolation

The PBMCs were rapidly thawed at 37^{o}C, diluted in complete culture medium (RPMI-1640 supplemented with 20% heat inactivated foetal bovine serum, 2 mM glutamine, 5% interleukin-2 (IL-2) and 50 µg/ml gentamicin), and centrifuged to remove the DMSO. Approximately 4 x 10^{6} cells were co-cultured in T-25 flasks (Corning) with 5 x 10^{6} PBMCs, pre-stimulated with 5 µg/ml phytohaemagglutinin (PHA) from a healthy HIV-seronegative blood donor. The

culture was incubated at 37^{O}C in an atmosphere of 5% CO_2 in air. For the first 48 hours, cells were cultured in complete medium containing 2 µg/ml polybrene, after which the culture was maintained in the same medium with no polybrene. Twice a week, half of the medium was removed and fresh PHA-stimulated PBMCs from a healthy HIV-seronegative donor were added to the culture. Aliquots of the culture supernatant were collected weekly for testing the reverse transcriptase (RT) activity and HIV-1 p24 antigen using Abbott's EIA. In addition, the culture was observed daily for cytopathic changes including cytolysis, giant cell and syncytia formation using 40x objectives in an inverted microscope.

After 50 days, several aliquots of cells from the original culture were cryopreserved in DMSO-medium and cell-free virus stocks were frozen in liquid nitrogen for further expansion of the virus *in vitro* and characterization of the molecular and biological properties.

Reverse Transcriptase Assay

Reverse transcriptase (RT) activity was measured as previously described (11) in the culture supernatants collected each week. Approximately 10 ml of the culture supernatant was clarified at 4^{O}C by centrifugation at 2,500g for 30 min. Cellular components were removed and the cell-free supernatant was centrifuged on a sucrose cushion at 100,000g for 60 min to concentrate particulate material. The high speed pellet was solubilized in 0.1 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 8.2, protease inhibitor (Leupeptin, Sigma), 0.05% Triton X-100, 0.1% glycerol, 160 mM NaCl, 2 mM dithiothreitol (DTT)) for 10 min. Supernatants from uninfected, PHA-stimulated PBMCs and known HIV-1 positive cultures were also included as negative and positive controls respectively. A pellet of murine leukaemia virus dissolved in the same solution was used as a test control. Enzyme activity was measured in 0.025 ml sample of the solubilized pellet using a reaction mixture containing 50 mM Tris-HCl (pH 8.2), $MgCl_2$, 8 mM DTT, 2 mM 3H-TTP (20 µCi) and 0.05 units per assay of template-primer (Poly(rA) oligo (dT)12-18. To test the specificity of the reaction, $MgCl_2$ was replaced by $MnCl_2$ in duplicate test samples using the same reaction mixture.

The reaction was carried out at 37^{O}C for 1 hr and terminated by quenching on ice. The product of the reaction was filtered through chromatography paper (Whatman No. 1) pre-soaked with TCA/Na-pyrophosphate/EDTA buffer. The filters were washed once with 10 ml of 5% TCA alone, four times in Na-pyrophosphate buffer, once with 5 ml of 95% ethanol and then dried under a heat lamp. Incorporation of radioactivity in dried filters was counted in Aquasol, a toluene-based scintillation fluid.

p24 Antigen Quantitation

Quantitation of virus production in co-cultivated cell cultures was also determined by assessing the amount of HIV-1 p24 antigen detected in culture fluids using the antigen capture EIA according the manufacturer's recommendations (HIVAG-1, Abbott Laboratories, Chicago, IL, USA). Six dilutions of known amounts of purified HIV-1 p24 antigen obtained as part of the Quality Assurance Programme sponsored by the National Institute of Allergy and Infectious Diseases were used as positive controls. Standard curves were constructed using a software program that allowed automatic calibration and quantitation of p24 values, expressed in picogram per ml of culture fluid.

The presence or absence of HIV-1 p24 in the plasma of the subject's blood was also tested and the amount was quantitated using the method described above, except that the control p24 dilutions were made in normal serum.

Identification of Viral Proteins by Western Blots

To identify immunological reactivities of the viral proteins, culture supernatants from the original co-cultivation were clarified and virus particles were concentrated through a sucrose gradient.

Virus pellets were disrupted in a buffer containing 2% sodium dodecyl sulphate (SDS), 10% glycerol, 0.07M Tris (pH 6.8) and bromophenol blue. Viral lysates were clarified by centrifugation at maximum speed in a microfuge for 10 min at $4^{o}C$. The supernatant was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the separated proteins were electroblotted on to nitrocellulose paper. Protein molecular weight markers (Sigma Chemical Company, St. Louis, MO, USA) and lysates of purified HIV (HTLV-IIIB strain) were run in parallel with the test sample as controls. Thin strips (2-3 mm) of the nitrocellulose paper containing the separated viral proteins were cut and incubated with 30 µl of the subject's plasma as described previously (11). Additional Western blot strips containing viral proteins from the Nigerian virus and those of HTLV-IIIB strain (HIV-1 control) were also reacted with known HIV-1 positive and HIV-negative sera. A second antibody conjugated to horseradish peroxidase and colour development was effected using a substrate as described previously (11). The patterns of reactivity in each blot were compared with the positive and negative controls for the identification of specific bands.

Host Range and Quantitation of Virus

In order to assess if the initial co-cultures of PBMCs from the Nigerian subject and the HIV-seronegative donor PBMCs were producing infectious particles, two human continuous T-cell lines (H9 and CEM), a monocytic cell line (U937), PBMCs from 6 different HIV-1 seronegative donors and freshly separated adherent macrophage monocyte cells were tested for their susceptibility to virus infection. The PBMCs were pre-stimulated with 5 µg/ml PHA prior to testing for infectivity and cultures were grown in complete medium. Since the T-cell line, (H9) and monocytic U937 cells were IL-2 independent, no IL-2 was added to the culture medium used for their growth. However, CEM cells were IL-2 dependent, hence growth media containing 5% IL-2 were used to culture the cells. Two weeks after the exposure of continuous cell lines (H9, CEM and U937) to virus, additional 5 x 10^5 cells of each cell line were added to their respective cultures on a weekly basis.

To obtain freshly isolated macrophage/monocyte cells from HIV-seronegative PBMCs, cells were allowed to adhere to the plastic surface for 2 hours and non adherent cells were discarded prior to virus exposure. Cultures were grown in the same medium as used for PBMCs.

The virus isolated from the Nigerian subject *in vitro* was quantitated on freshly isolated PBMCs, macrophages or cell lines in 24-well plates (Table 2). Seven serial 5-fold dilutions of cell-free culture supernatants were tested in each cell type.

For each cell type, 2 x 10^6 cells were enumerated, and duplicate sets of cultures were incubated with 100 µl of cell-free virus (supernatant from the original cultures), for 1 hr at $37^{o}C$ in 5% CO_2. After 1 hr, the virus was removed by centrifugation and cells were re-suspended in 2 ml of the growth medium and incubated at $37^{o}C$ in 5% CO_2 atmosphere. Twice a week, approximately half of the cell-free supernatant was replaced with a fresh medium containing 5 x 10^5 PHA-pre-stimulated PBMCs to maintain sufficient supply of susceptible cells in the culture.

Cultures were monitored daily for cytopathic effects including single cell death, as well as giant cell and syncytia formation. Aliquots of culture supernatants were collected weekly and tested for HIV-1 p24 antigen as described above.

Phenotypic Analysis

The syncytium (SI) or non-syncytium including (NSI) phenotype of HIV-1_{IbNg} was tested in MT-2 cells as described previously (12). Briefly, 12 wells of a 96-well microtiter plate were seeded with 5 x 10^4 MT-2 cells in 150 µl RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% foetal bovine serum. Cells in 6 of the 12 wells were infected

with 50 µl of the test culture supernatant. In two experiments, cells/supernatant mixture from previously positive cultures of the Nigerian virus were plated on MT-2 cells. Three wells of MT-2 cells received 50 µl of culture medium as negative controls and the other three were infected with a known SI-inducing HIV-1 strain as positive controls. Cultures were incubated in an atmosphere of 5% CO_2 in air and examined daily for syncytia and other cytopathic changes using 40x objective in an inverted microscope.

Genotypic Analysis

To identify the genotype of the virus isolated from Nigeria, we obtained 60 cDNA clones of PCR amplified products of HIV-gene. Fourteen of these clones containing the envelope gene (gp120) were analyzed for DNA nucleotide sequences (13).

RESULTS

The plasma from the Nigerian subject was positive for HIV-1 antibodies. No HIV-2, HTLV-1 or HTLV-2 antibodies were detected. The plasma also contained 125 pg/ml of HIV-1 p24 proteins.

Recovery of HIV-1 Isolate

Co-cultivation of cryopreserved PBMCs from the asymptomatic Nigeria blood donor with PHA-stimulated PBMCs from an HIV-1 seronegative donor resulted in the isolation of a retrovirus related to HIV-1. This virus has been designated as HIV-1_{IbNg}. Replication of HIV was initially detected by RT activity after 4 weeks of co-cultivation. This activity increased significantly in the 5th week of culture, at which time HIV-1 p24 antigen was also detected in the culture fluid. The reverse transcriptase activity present in the culture supernatants ranges from 5.4×10^5 cpm on day 35 to 1.4×10^6 cpm on day 56; whereas levels of detectable HIV-1 p24 antigen ranged from 30 pg/ml on day 24 to 2,000 pg/ml on day 56.

A significant number of single cells died within the first 10 days of co-cultivation. Therefore, in order to maintain virus production in the culture of PHA-stimulated healthy, HIV-seronegative PBMCs were added daily or bi-weekly. Although ballooning of PBMCs and giant cell or syncytia formation were not observed in the initial co-cultivation, cytopathic effects and cytolysis of single cells appeared concomitantly with the detection of high levels of RT activity and HIV-1 p24 antigen in the culture supernatant.

Immunoreactivity of HIV-1_{IbNg} Proteins

The HIV-1_{IbNg} proteins were prepared from the virus pelleted by ultracentrifugation (100,000g) of cell-free supernatant of the original culture and blotted on nitrocellulose paper. Viral proteins were identified by reacting immunoblots (Western blots) with the autologous serum/plasma antibodies. The pattern of immunoreactivity of the HIV-1_{IbNg} proteins was similar to that seen in the HIV-seropositive control sera reacted with an immunoblot of a known American isolate, HIV-1 (HTLV-IIIB). Reactive bands detected in the HIV-1_{IbNg} immunoblots using autologous and HIV-positive control sera included gag-encoded p18, p24, pol-encoded p31, p55, p66 and env-encoded gp41 and gp120 proteins. Sera from HIV-1 negative donors did not react with proteins from the Nigerian virus isolate or HIV-1 (HTLV-IIIB) controls.

Host Range, Biological Properties and Quantitation of the Virus Isolate

Six different PBMC cultures from HIV seronegative donors were tested for susceptibility to virus infection. Cells exposed to various HIV-1_{IbNg} virus stocks harvested at various intervals during the co-cultivation showed evidence of virus replication within 3-4 days post-infection and both the p24 antigen and RT activity were detected in all cell cultures. Cytopathic changes consisted primarily of single cell death and were observed within 1 to 4 days of infection, depending on the concentrations of the virus stocks used. With a multiplicity of infection of 0.1, cell

death in PBMCs was observed within 24 hours and the virus-growth could only be sustained in these cultures by adding freshly stimulated PBMCs everyday. The HIV-1_{IbNg} replicated to t titre of 2.5 x 10^2 cell culture infectious units ($CCIU_{50}$) per ml in 6 days and cell death progressed faster in the PBMCs than in the established cell lines (Table 2). This necessitated in media change with the addition of freshly stimulated PBMCs at least twice a week if not daily. The freshly isolated adherent monocytes/macrophages from healthy individuals were highly susceptible to infection (1.25 x 10^3 $CCIU_{50}$ per ml) and showed insignificant cytopathic effects (Table 2).

Table 2: In vitro host range of HIV-1_{IbNg}

Cell Type	$CCIU_{50}$/ml**	Cytopathic Effects***
Peripheral blood Mononuclear cells*	2.5 x 10^2	4+
Macrophages/Monocytes*	1.25 x 10^3	1+
T-cell lines		
H9 (IL-2 independent)	2.5 x 10^2	3+
CEM (IL-2 dependent)	1.25 x 10^3	3+
Monocytic cell line		
U937	2.5 x 10^2	3+
HTLV-I infected T-cell line (MT2)	-	3+

* PBMC and adherent cells from six different donors.

** Cell culture infectious unit 50% (CCIU50) is defined as the reciprocal of virus dilution yielding infectious virus in 50% of cultures 6 days after the exposure to HIV-1_{IbNg}.

*** Cytopathic effects are defined as single cell death, very few or no giant cell syncytia formation were observed.

The three cell lines (CEM, H9 and U937) were also susceptible to the replication of HIV-1_{IbNg} *in vitro* with observable reverse transcriptase activity and HIV-1 p24 antigen in all culture supernatants tested on day 14. The highest levels of both p24 and RT activity were found in CEM cells, (1.25 x 10^3 $CCIU_{50}$/ml) followed by U937 and H9 cells (2.5 x 10^2 $CCIU_{50}$/ml). The virus also induced cytopathic changes in all three cell lines but with varying degrees of cytolysis (Table 2). Cell death was observed faster, commencing from the second week in H9 and CEM cells but delayed until week 3 in U937 cells. Formation of syncytial cells was rare but single cell death and cell fusion occurred earlier in H9 and CEM cell lines than in U937 cells.

Phenotypic Analysis of HIV-1_{IbNg}

The phenotypic analysis indicated that HIV-1_{IbNg} did not induce typical syncytia seen in MT-2 cells inoculated with SI isolates of HIV-1 used as controls. The balloon-shaped giant cells were rare and individual cell death was observed 3 to 4 days after infection. Most HIV-1_{IbNg} exposed MT-2 cells died after 10 days.

Genotype Analysis

A total of 14 clones containing the *env* gene were analyzed for the DNA nucleotide sequences of gp120. Eight clones containing the 5' end and 6 clones

containing the 3' end were sequenced. Comparison of gp120 encoding nucleotide sequences and deduced amino acids of HIV-1_{IbNg} with other known HIV-1 isolates placed this virus in a distinct category of HIV-1 subtype A, a clade which has been shown to occur predominantly in West Africa (12,13).

DISCUSSION

We have identified a new subtype A HIV-1 strain (HIV-1_{IbNg}) from an asymptomatic blood donor. This is the first report of an HIV-1 isolate from Nigeria. Immunological reactivities of the major structural proteins of HIV-1_{IbNg}, as determined by Western blot analysis, are similar to those observed in the known HIV-1 isolates which have been described from other parts of the world (8,14). The RT activity of this virus is Mg^{2+}-dependent and is produced in high quantities. The p24 antigen detected in culture supernatants is HIV-specific.

The somewhat longer time ($\geq$ 50 days) for the continuous growth of HIV-1_{IbNg} *in vitro* was probably due to the extreme cytopathic effects observed within the first week of the Nigerian subject's PBMCs. Since freshly stimulated PBMCs were added only once a week to the initial co-culture, it is possible that most of the virus released in the supernatant was "lost" because sufficient number of pre-stimulated PBMCs were not available for the newly released virus to initiate new infectious cycle. Although this phenomenon is observed in many highly cytopathic viruses, it is also possible that the extreme cytotoxicity was enhanced initially due to differences in histocompatibility antigens between the PBMCs of the Nigerian subject and those used for co-cultivation (S. Rasheed, unpublished observation).

Although HIV-1_{IbNg} was isolated from an asymptomatic male, he was admitted to the hospital for an ailment within 6 months after donating the blood which yielded the HIV-1_{IbNg} isolate. This patient was managed at a private hospital for four months and during this time his medical records showed he was occasionally febrile, progressively wasting, dehydrated and had oral thrush. He had generalized dermatoses, prominent herpetic angular stomatitis, gingivitis and glotitis. No other opportunistic infections were recorded. His most severe complication was anaemia. The patient had not received any antiviral medication. He was discharged from the hospital at the request of his family because of poor response to treatment. Two weeks later he lapsed into coma and died at home. The development of clinical AIDS in this patient only a few months after blood donation indicates that the fast-replicating virus may have induced cytopathic effects and depletion of CD4+ cell *in vivo* which may have enhanced the progression of the disease. An alternative explanation is that the virus may possess some yet unidentified and unique molecular properties or sequence variations that may have promoted the observed pathogenesis (15).

HIV-1_{IbNg} has a broad host range and replicates well in both IL-2-dependent T-cell line (CEM) and IL-2-independent T-cell line (H9) as well as monocytic cell line (U937). Healthy, PHA-stimulated PBMC cultures from several HIV-seronegative donors separately exposed to different stocks of HIV-1_{IbNg} yielded infectious virus within 3 days and showed significant single-cell cytopathology. This virus also efficiently infects freshly harvested adherent monocyte/macrophage cells *in vitro* with the least cytopathic effects. This is important because the majority of macrophage-tropic HIV-1 isolates, with a few exceptions, have been isolated from the central nervous system and are not cytopathic to T-cells (16-20). A similar cell tropism was reported for the virus isolated in Philadelphia from a Jamaican patient with AIDS (21). Thus, the isolation of a highly cytopathic, macrophage-tropic strain from PBMCs of an apparently asymptomatic individual without neutrologic disease implies the HIV-1_{IbNg} is responsible for the rapid onset of the illness in this person. Studies of 'atypical' viruses would lead to a better understanding of HIV pathogenesis.

Variability in the envelope region has been suggested as the most likely explanation for the difference in infectivity and syncytium-inducing capacity of HIV-1 isolates in MT-2 and other cells (22-25). Furthermore, highly cytopathic and fast replicating HIV-1 strains that readily infect both T- and monocyte cell lines have been isolated from patients with full-blown AIDS

and are associated with high mortality rate (7,14,26). Thus, syncytium-inducing isolates have been reported more often in patients with AIDS than in asymptomatic seropositive individuals (5-7,14). It has also been proposed that the occurrence of a particular virus variant may correlate with the clinical course of the disease suggesting that *in vitro* biological properties of HIV-1 may be relevant to at least some *in vivo* situations (27). HIV-1_{IbNg} does not induce typical syncytia in MT-2 cells but is highly cytopathic in PBMCs and in both T-cell and monocytic cell lines.

Many variable regions in HIV gp120, DNA nucleotides and amino acid sequences of the V-3 loop have been used to classify different regional subtypes or clades of HIV-1 isolates (8). Although a comparison of sequences of the HIV-1_{IbNg} go120 V-3 loop with known HIV-1 isolates classifies this virus within the HIV-1 subtype or clade "A", when the sequences of the entire gp120 are compared with other isolates, the HIV-1_{IbNg} falls into a distinct category of HIV-1 subtype "A" viruses (13).

Although the length of time between HIV-infection and the diagnosis of AIDS in the Nigerian donor is not known, it can be speculated that this virus is highly pathogenic because of the clinical onset of the disease within a short period. Furthermore, the broad host range and unusual biological properties of HIV-1_{IbNg} together with unique mutations in the gp120 gene suggest that this virus strain may be directly involved in the pathogenesis. Mutational studies using infectious molecular clones of HIV-1_{IbNg} *in vitro* may lead to a better understanding of the mechanism(s) by which this virus may have caused a rapid progression to AIDS in the infected individual.

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