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Molecular cloned SHIV-CN97001: a Replication-Competent, R5 Simian-Human Immunodeficiency Virus Containing Env of a Primary Chinese HIV-1 Clade C isolate

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Abstract

The increasing prevalence of human immunodeficiency virus type 1 (HIV-1) subtype C infection worldwide calls for efforts to develop a relevant animal model for evaluating AIDS candidate vaccines. In China, the prevalent HIV strains comprise a circulating recombinant form, BC (CRF07_BC), in which the envelope belongs to subtype C. To evaluate potential AIDS vaccines targeting Chinese viral strains in nonhuman primate models, we constructed a simian-human immunodeficiency virus (SHIV) carrying most of the envelope sequence of a primary HIV-1 clade C strain isolated from an HIV-positive intravenous drug user from YunNan province in China. Infection of six Chinese rhesus macaques with SHIV-CN97001 resulted in a low level of viremia and no significant alteration in CD4⁺ T-lymphocyte counts. To determine whether in vivo adaptation would enhance the infectivity of SHIV-CN97001, the parental infectious strain was serially passaged through eight Chinese rhesus macaques. The hallmarks of SHIV infectivity developed gradually, as shown by the increasingly elevated peak viremia with each passage. These findings establish that the R5-tropic SHIV-CN97001/Chinese rhesus macaque model should be very useful for evaluation of HIV-1 subtype C vaccines in China.

Keywords

Clade C; Passage; Rhesus macaques; SHIV

Introduction

Among the major circulating HIV-1 subtypes, subtype C is the most prevalent and is responsible for much of the aggressive HIV epidemic in China. It is indicated that about 0.74 million people living with HIV/AIDS in China by the end of 2009 (37). A high proportion of them are infected with HIV-1 CRF07_BC strains, in which the envelope gene is mainly derived from the Indian clade C virus (7, 28, 32). Up to now, several candidate

vaccines in China have been developed, mainly containing genes from CRF07_BC strains (6).

Efforts to understand HIV-induced disease and to develop an effective vaccine against HIV-1 will require animal models. The simian immunodeficiency virus (SIV)/macaque model is of limited utility because of a lack of significant diversity of the *env* gene (21). To address this limitation, chimeric simian-human immunodeficiency viruses (SHIVs) containing the *tat*, *rev*, *vpu*, and *env* genes of HIV-1 have been constructed and shown to infect macaques (1, 26, 31, 35, 38). Most current SHIV constructs utilize envelope genes derived from HIV-1 subtype B strains, either from lab-adapted strains (HIV-1 HXB2 and HIV-1 NL43) or from primary, CCR5-tropic (HIV-1 162), CXCR4-tropic (HIV-1 33), and dual-tropic (HIV-1 89.6 and HIV-1 DH12) isolates (16, 22, 25, 33). Even though HIV-1 clade C and other non-B clade viruses are responsible for the majority of AIDS cases globally, only a few animal models that utilize viruses from these sources exist for studies of pathogenesis and vaccine development (2, 10, 19, 20).

Five SHIV strains (SHIV-CHN19, SHIV-MJ4, SHIV-MCGP1.3, SHIV-XJ02170 and SHIV-1157ipd3N4) encoding the envelope gene of HIV clade C have been created thus far (2, 3, 27, 34, 39). Here we report the construction of a SHIV that expresses most of the *env* gene of a primary Chinese HIV-CN97001 (30), which was isolated from YunNan province in China by the China CDC and the European Commission-funded research cluster CHIVAC to generate region-specific candidate vaccines. This strain and subsequently detected, closely related variants represent at least 60% of the HIV infections in China and are also antigenically close to other C clade isolates from India and South and East Africa. Recently, HIV-CN97001 *env* gene was proved to induced high level of neutralization antibody (8). In this study, SHIV-CN97001/Chinese monkey model was constructed to evaluate the homologous immune protective efficacy of Chinese AIDS vaccine candidates.

SHIV constructs always require in vivo adaptation and often serial in vivo passage to acquire the ability to replicate to high titers and induce simian AIDS in macaques (14). Up to now, the vast majority of AIDS vaccine/challenge data have been generated in rhesus macaques of Indian origin. However, the supplies of Indian macaques are limited (4). Chinese rhesus macaques, both wild and captive, can be supplied in much larger numbers than Indian macaques, and therefore could be used as substitutes for Indian macaques. In this study, SHIV-CN97001 was constructed and passaged in Chinese monkeys to enhance its infectivity.

Materials and methods

Amplification of HIV-1 *env* using a nested PCR method

The specimen for this study was isolated from peripheral blood mononuclear cells (PBMC) obtained from an intravenous drug user infected with HIV-1 in YunNan, China. High molecular-weight genomic DNA was extracted from PBMC using a DNeasy genomic DNA isolation kit (Qiagen, Valencia, CA, USA). To amplify a 1.7-kb HIV-1 gene fragment containing gp120 and partial gp41 sequences (covering N-terminal amino acids 50–650), nested primers were designed according to conserved regions of the HIV-1 clade C *env* gene with the addition of restriction enzyme sites for KpnI or AvrII in the internal primers. The two external primers were: CGAGAAGAAGCGGAGACAGCGACGAA (sense) and AGCCAGGCACAAGCAGCATTGTAGC (antisense); and the two internal primers were CACAGTCTATTATGGGGTACCTGTATGG (KpnI site underlined) and ATCCCTAGGAGCTGTTGATCCTTTAG (AvrII site underlined). The first round PCR was run under the following conditions: one cycle at 94°C for 2 min; then 35 cycles at 94°C for 30 s, 60°C for 30 s with a touchdown of 0.3°C per cycle, 72°C for 3 min, and a final

extension at 72°C for 6 min. For the second-round PCR, 1 µl of first-round PCR products was used as template, and the PCR conditions were: one cycle at 94°C for 2 min; then 34 cycles at 94°C for 30 s, 54°C for 30 s, 72°C for 2 min; and followed by a 72°C extension for 6 min.

Genetic analysis and phenotypic characterization of the amplified HIV-1 genes

1.7-kb *env* amplicons were cloned into the vector pMD18-T Simple (Takara, China). The fragments were sequenced by using an automated DNA sequencer (ABI Prism 377). Multiple sequence alignment was carried out using the PileUp program in the GCG package [Wisconsin Package Version 10.2; Genetics Computer Group (GCG), Madison, WI, USA]. Phylogenetic analysis was performed using the Phylogenetic Analysis Using Parsimony (PAUP*) program (version 4.0) and the GCG programs PAUPSearch and PAUPDisplay. The PAUPSearch program used the analysis option 'Heuristic Tree Search' and the Parsimony Optimality Criterion for tree search. The *env* sequences for all clades were obtained from the Los Alamos HIV Sequence Database (<http://hiv-web.lanl.gov>; accessed 28 February 2005).

Construction and characterization of SHIV clones

Two plasmids, 5'SHIV-KB9 and 3'SHIV-KB9, were used as the starting backbones for the construction of SHIV-CN97001. First, a unique restriction site, *AvrII*, was introduced into the gp41 gene in 3'SHIV-KB9 by the toxic protein selection mutagenesis method (40). The *AvrII* site was located in the DNA sequence encoding the immunodominant region of gp41. Then, a 1.7-kb *KpnI-AvrII* gene fragment encoding the HIV-CN97001 envelope (covering N-terminal amino acids 50–650) was amplified by PCR using genomic DNA as template (extracted from the whole blood of patient CN97001) to replace the corresponding region of 3'SHIV-KB9 *env* (Fig. 2). Nested primers were designed according to conserved regions of the HIV-1 clade C *env* gene with the addition of restriction enzyme sites for *KpnI* or *AvrII* in the internal primers. The resulting 3'SHIV-CN97001 was linearized by *SphI* and *NotI* double digestion, and then ligated with the 5' half of SHIV-KB9 previously treated with *SphI* and *NotI* to obtain the full-length SHIV-CN97001 proviral clone.

Coreceptor utilization assays

Ghost-CD4-CXCR4 and Ghost-CD4-CCR5 cell lines carry the HIV-2 long terminal repeat-driven green fluorescence protein (GFP) gene, which becomes activated upon infection with HIV or simian immunodeficiency virus. Viral entry is followed by Tat activation of transcription and GFP becomes expressed. Cells express GFP upon infection with SHIV strains that use the CXCR4 or CCR5 coreceptor. The cells were seeded in 24-well plates 24 hours prior to infection, 2×10^5 cells per well. Then cells were infected overnight with 10 ng of p27gag of each virus stock per ml. Medium was replaced the following morning. After 60-hour cultivation, the cultures were photographed by phase-contrast microscopy to detect fluorescence. Then, cells were washed, trypsinized, and resuspended in 200 µl of phosphate buffered saline (PBS) containing 2% serum, and 20000 cells were evaluated by flow cytometry. Each experiment was performed in triplicate.

Animal inoculation

Fourteen Mamu-A*01 negative Chinese-origin rhesus macaques (*Macaca mulatta*) were used in this study, and divided into two groups. All of the animals were seronegative for SIV, simian retrovirus type D (SRV), simian T-cell leukemia virus (STLV) and herpes B virus. Six animals in group 1 were used to determine the 50% monkey infectious dose (MID50), and eight in group 2 for in vivo passage. The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee at the Kunming Institute of

Medical Biology and Institute of Laboratory Animal Science, where the experimental animals were housed. For MID50 titer, 6 monkeys were intravenously (i.v.) inoculated with different doses of cell-free viral supernatant, 0.2–200 TCID₅₀ in 10-fold increments. For the rapid animal-to-animal passages, each of P1 animals were intravenously infected with 200 TCID₅₀ of SHIV-CN97001 cell-free viral supernatant. Then, 10 ml of whole blood collected from each of the P1 monkeys at 4 weeks postinfection (p.i.) was transfused intravenously into two P2 macaques, separately. Subsequent passages for P3 animals were carried out in the same manner as for P2 animals except for increasing the number of animals.

Plasma viral RNA quantitation

Quantitative assays for the measurement of SHIV-CN97001 RNA were performed by real time RT-PCR using primers and probe specific for SIV *gag* in an ABI Prism 7700 sequence detection system (PE Applied Biosystems, Foster City, CA, USA) as previously described(13). Briefly, viral RNA from 0.2 ml of EDTA-anticoagulated cell-free plasma was directly extracted by a MagNa Pure LC robotic work station (Roche Molecular Biochemicals). One-step reverse transcriptase PCR (RT-PCR) using the TaqMan EZ RT-PCR CORE REAGENT kit was performed to quantify SHIV viral RNA. This assay has a threshold of quantification of 200 copies/ml of plasma.

FACS analysis

CD4⁺ T-cell percentages were derived by flow cytometric analysis of blood lymphocytes using a whole-blood lysis technique (CD3-FITC, cat.556611, CD4-PerCP, cat.550631 and CD8-PE, cat.557086, BD Pharmingen, San Diego, CA, USA) and examined on a FACSscan analyzer (Becton-Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's recommendations. Absolute lymphocyte counts in the blood were determined with an automated blood cell counter (F-820; Sysmex).

Statistical analysis

Plasma virus levels were transformed to log₁₀ copies/ml prior to all analyses. Peak viral load was the maximum value measured between days 7 and 35 inclusive. Differences between groups were tested using one-way analysis of variance. Statistical significance was defined as $P < 0.05$.

Results

Sequence analysis of HIV-CN97001 *env*

Before SHIV construction, copies of the 1.7-kb fragment amplified to replace the *env* gene of SHIV-KB9 were cloned into plasmid pMD18-T Simple vector and subsequently sequenced. Comparison of this 1.7-kb fragment with the entire gp160 sequence of various well-known genetic subtypes of HIV identified HIV-CN97001 as a member of HIV-1 subtype C (Fig. 1). The *env* gene from HIV-CN97001 clustered tightly within the subtype C subfamily by a neighbor joining method. HIV-CN97001 is closely related to a Chinese primary isolate, 98CN009 (GenBank accession number AF286230), which was characterized as a CRF_07BC subtype. The limited genetic distance of HIV-CN97001 *env* from other Chinese C strains makes it a representative virus of the subtype C strains that are now prevalent in China.

Construction of replication-competent SHIV-CN97001

The *env* gene was directly amplified from PBMC of a Chinese intravenous drug user patient without any in vitro manipulation. Nearly 20 clones were constructed and each construct

was analyzed by restriction enzyme digestion and further validated by DNA sequencing (Fig. 2). The verified plasmid was transfected into 293T cells for infectious SHIV production. SHIV-CN97001 replicated well in PBMC of human and multiple monkeys, in which p27 production ranged from 1 to 40 ng/ml in the culture supernatants 12 days postinfection (data not shown). The 50% tissue-culture infective dose (TCID₅₀) of the virus stock was confirmed in TZM-bl cells. The virus stock was diluted and used to infect cells. In this study, one TCID₅₀ of SHIV-CN97001 in TZM was 10 µl of virus stock.

The backbone SHIV-KB9 and the new chimera SHIV-CN97001 were examined for their coreceptor usage by using GHOST cells expressing CXCR4 or CCR5. The backbone SHIV-KB9 used both CXCR4 and CCR5, whereas SHIV-CN97001 used only CCR5 as a coreceptor (data not shown). Therefore, the coreceptor usage of each SHIV is consistent with that of its parental HIV-1.

Coreceptor usage of SHIV-CN97001

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Infectious activity in Chinese monkeys

To evaluate in vivo infectivity, six Chinese monkeys were intravenously infected with 0.2–200 TCID₅₀ virus stocks, in 10-fold dilutions. Plasma viral RNA loads and CD4/CD8 T-cell ratios were monitored longitudinally for 98 days with more frequent sampling in the first 4 weeks of the infection. All the three monkeys with 200 or 20 TCID₅₀ virus and only one inoculated with 2 TCID₅₀ virus became infected, indicating that the 1 MID₅₀ in Chinese rhesus macaques was 20 µl of virus stock (Fig. 3E). However, the data showing only one animal may have a slightly lower CD4/CD8 ratio (Fig. 3D).

Serial passages in Chinese rhesus macaques

To determine whether in vivo adaptation would enhance the infectivity of SHIV-CN97001, passages were carried out serially in three groups, via intravenous blood transfusion. Both P1 animals (M07 and M08) developed a productive infection characterized by a burst of viral replication during the initial 1 to 3 weeks after intravenous inoculation (Fig. 4A). In comparison to P1 macaques, M09 and M10 had increased median peak viral loads from 10^{5.84} to 10^{6.15} copies of viral RNA per ml of plasma. Further successive passages significantly enhanced viral infectivity, as shown by viral median peaks of 10^{6.60} copies of viral RNA per ml of plasma in four P3 macaques. Statistical analysis showed that viral load increase upon passaging was significant ($P < 0.05$) (Fig. 4D). Furthermore, there was a faster kinetic in the increase in viral RNA copy upon passaging. The viral load in all macaques became undetectable 5 months postinfection and only one P3 animal [M11] rebounded 1 year later to a very low level of viral load for about 1 month, which then diminished again (Fig. 4C).

All P1 to P3 animals became seropositive 3 to 4 weeks postinoculation and serial passages did not change the viral phenotype, as confirmed by the persistence of the R5 tropism of SHIV-CN97001 isolated from P3 animals. Meanwhile, no significant CD4 lymphopenia was found in any infected animal even 2 years after transfusion (data not shown).

No evidence for animal-specific viral change in env during passage

At least six cDNA sequences were cloned from each animal on the 3rd week after infection. From each time point, at least 2800 bp in *env* region of data were obtained. Relatively few

changes were seen in the dataset of 24 sequences: the consensus of the sequences in P3 differed from the SHIV-CN97001 input sequence at only two nucleotides (causing missense mutations A160V and V297I).

DISCUSSION

To prevent an HIV/AIDS epidemic in China, a number of HIV candidate vaccines have been developed, necessitating the establishment of suitable animal models to evaluate vaccine efficacy and safety. In this study, a novel chimeric SHIV expressing envelope glycoproteins cloned from a primary R5-tropic clade C Chinese HIV-1 isolate was generated and shown to be infectious in rhesus macaques. In the Chinese National HIV Molecular Epidemiology Study (NHMES), which began in the mid 1990s, more than 3000 HIV samples were sequenced and analyzed. By 2003, the prevalence of the HIV-1 B*/C clade had increased dramatically, comprising 55% of all HIV-1 clades found to have spread to 19 of 31 provinces (17). Thus, the *env* gene encoded by our new SHIV strain belongs to the most prevalent HIV circulating recombinant form in China.

SHIV-CN97001 is one of only a few CCR5-utilizing SHIVs that have been constructed so far. The first R5 SHIV, SHIVSF162P3, encodes *env* of a primary, macrophage (M)-tropic HIV clade B strain; it has been shown to intravaginally infect rhesus monkeys, half of which progressed to AIDS 24–44 weeks after inoculation (9). Two other R5 SHIVs are SHIVCHN19 (3) and SHIV-XJ02170 (39), which express most of the envelope sequences from two different primary HIV clade C isolates in China. SHIV-CN97001 was found to replicate in human PBMC at a higher level than SHIV-XJ02170 (data not shown).

We performed three rapid in vivo passages of SHIV-CN97001 in eight Chinese rhesus macaques to generate variants that were more replication-competent. Generally, the passages greatly enhanced the infectivity of the virus, as shown by the increasingly elevated viral loads during acute infection in animals with each passage. First, compared to the P1 virus recipient, the subsequent animals had peak viral RNA loads that were 0.5–1 log higher. Second, the speed of viral replication was also greatly enhanced, as evidenced by the shortened viral doubling time from P1 (1.4 to 1.6 days) to P3 (0.7 to 0.8 day) animals (data not shown). The finding that these later-passage viruses appeared to replicate with better efficiency suggests the evolution of variants with increased replication capacity in the new host. Previous studies provided direct evidence that amino acid changes in the HIV-1 envelope glycoproteins were responsible for either increased replication capacity or the CD4⁺ T cell-depleting ability of pathogenic SHIVs (5, 18). We analyzed sequences of the original virus clone and those of all animals used for passage. Comparison of the *env* cDNA sequences of the passaged SHIV-CN97001 viruses with that of the parental virus revealed an alanine-to-valine change in the V2 region and a valine-to-isoleucine substitution in C2 of the gp120 glycoprotein. Moreover, the gp120 RNA sequences analysis revealed that the genetic distances of SHIV-CN97001 in the third passage animals were the highest during in vivo passage (23). These changes might have contributed to the capacity of the passaged viruses to replicate efficiently in vivo.

Despite robust replication of SHIV-CN97001 through in vivo passages, no significant CD4 lymphopenia was found in the blood of infected animals even 2 years after transfusion. Numerous studies in HIV-1-infected humans and SIV-infected monkeys have suggested that efficient virus replication is a prerequisite for CD4⁺ T-lymphocyte destruction and viral pathogenicity. The 5- to 10-fold increase in acute viremia seen for the passaged SHIV-CN97001 may not allow the pathogenic process to impact a large percentage of the CD4⁺ T-lymphocyte population. Moreover, as to the peripheral blood, there was no CD4 lymphopenia in intestinal mucosa in passage 3 monkey (M11). Further work will be

required to increase the peak viremia of SHIV-CN97001. Otherwise, differences in coreceptor usage can influence the pathogenic outcome in SHIV-infected monkeys (2). Recently, Cheng-Mayer and colleagues described for the first time a coreceptor switch of an R5 SHIV-B, SHIVSF162P3N (11, 12, 36). Coreceptor switching from CCR5 to CXCR4 is observed in approximately 50% of HIV-B-infected humans but only rarely in HIV-C-infected individuals, which is accordance with the findings that no coreceptor switch occurred in SHIV-CN97001 during in vivo passage.

A possible reason why pathogenic mutant strains of SHIV-CN97001 did not appear during passage might be the existence of different immune pressures between Chinese-origin and Indian-origin rhesus macaques. Although Chinese monkeys and Indian monkeys are phylogenetically related, they differ significantly in their susceptibility to AIDS-related disease induced by infection with SIV or SHIV. Preliminary observations by our group suggest that SHIV-KB9 is less pathogenic in Chinese macaque subspecies (24). Moreover, the immune systems of the selected monkeys in this study had already matured. It may be that an ideal effect could be obtained if juvenile rhesus macaques were used, because previous studies showed that long-term adaptation of viruses in juvenile rhesus monkeys and rapid passage can induce the emergence of pathogenic variants (14). Finally, it is necessary to include the bone marrow for transfusion, based on an earlier finding that some virulent viral strains might reside there (15).

Up to now, there is not a pathogenic SHIV virus strain containing env gene from a primary clade C Chinese HIV-1 isolate. So, it is not possible to evaluate the China's AIDS vaccine candidates homologous protection from disease. However, the nonpathogenic SHIV-CN97001/Chinese monkey model could be used to evaluate the vaccine protection efficacy from intravenous infection by acute viremia analysis. We anticipate that the final adaptation of our new virus will not be completed for some time, as we plan to continuously passage the virus in monkeys until a new, pathogenic clade C SHIV, is obtained.

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Fig. 1. Phylogenetic analysis of the HIV-1 CN97001 *env* gene from China. A neighbor-joining phylogenetic tree was built using the F84 model in the Phylip software package (V3.68). Significant bootstrap values (>70%) are placed next to the nodes. The genetic distance corresponding to the lengths of the branches is shown by the scale below the tree. Reference samples are indicated and were obtained from the Los Alamos National laboratory HIV database (<http://hiv-web.lanl.gov/>).

SHIV89.6P(KB9)

SHIV CN97001

Fig. 2.

Schematic illustration showing construction of SHIV-CN97001. A unique restriction site, *AvrII*, was first introduced into the 3' half of SHIV89.6P, clone KB9 (29), proviral DNA. The 1.7-kb *KpnI-AvrII* fragment of HIV-CN97001 was amplified to replace the corresponding region of KB9 *env*. Finally, the modified 3' half containing CN97001 *env* was ligated with the 5' half of KB9 to form the full-length SHIV-CN97001 construct.

Fig. 3. Plasma viral RNA loads and T-cell subsets in rhesus monkeys inoculated with SHIV-CN97001. The six animals used for virus TCID₅₀ titration were divided into 4 groups: A, C, E, G, plasma viral RNA loads; B, D, F, H, CD4/CD8 ratios. The threshold of detection of viral RNA was 200 copies/ml. Animals receiving the same dose are plotted in the same panel, as follows: one 200TCID₅₀ (M01), two 20TCID₅₀ (M02 and M03), two 2TCID₅₀ (M04 and M05), and one 0.2TCID₅₀ (M06) macaques.

Fig. 4.

Changes in plasma viremia over time in P1 to P3 rhesus macaques postinfection. Animals from the same passage are plotted in the same panel, as follows: two P1 (M07 and M08, upper left), two P2 (M09 and M10, upper right), and four P3 (M11, M12, M13 and M14, lower left) macaques. The peak viral RNA copies per milliliter of plasma in the peripheral blood of these monkeys are shown in (D).

Table 1

Coreceptor usage of parental and chimeric viruses

Virus	GHOST cell	
	CXCR4	CCR5
SHIV-KB9	++	+
SHIV-CN97001	–	+
Mock-infected	–	–

* Co-receptor usage was determined after infection of GHOST cells expressing CXCR4, CCR5 with virus stocks. These experiments were performed at least three times with similar results. “–”: <2%; “+”: 2%–5%; “++”: 6%–20%; “+++”: >20%.