第19卷2期
2004年4月

中国病毒学
VIROLOGICA SINICA

19（2）：114—119
April 2004

全基因组SHIV-KB9克隆的构建及其在人、猴外周血单核细胞中的复制*

吴颖运[1,2]，洪坤学[2]，陈 佩[2]，陈启明[1]，耿运琪[1]，邵一鸣[1,2**]

（1. 南开大学生命科学院，天津 300071； 2. 中国疾病预防控制中心性病艾滋病预防控制中心，北京 100050）

摘要：将分别携带SHIV-KB9 (SIV/HIV-1 KB9) 基因组的3′端和5′端的两个半长克隆，体外连接成SHIV-KB9全基因组克隆。含有全长基因的质粒培养时易发生同源重组和缺失，采用JM109作为宿主菌以及30℃、低转速的培养条件，可保持质粒的稳定性。通过PCR，RT-PCR和猴免疫缺陷病毒(SIV) *gag* p27核心抗原滴度检测表明：感染性克隆SHIV-KB9可有效在人、恒河猴及食蟹猴的外周血单核细胞中复制。

关键词：SHIV-KB9；全基因组；感染；PBMC

中图分类号：Q78　　**文章标识码**：A　　**文章编号**：1003-5152(2004)02-0114-06

Generation of Full-genome SHIV-KB9 Clone and Its Replication in Human and Monkey Peripheral Blood Mononuclear Cells*

WU Ying-yun[1,2], HONG Kun-xue[2], CHEN Pei[2], CHEN Qi-ming[1], GENG Yun-qi[1], SHAO Yi-ming[1,2**]

(*1. College of Life Sciences, Nankai University, Tianjin* 300071, *China*; *2. National Center for AIDS/STD Control and Prevention, Beijing* 100050, *China*)

Abstract: SHIV-KB9 chimeric virus was constructed by two constructs representing the 3' and 5' fragments of the viral genome. This full-genome SHIV-KB9, with large size and long terminal repeat sequence (LTR), could be maintained stable in *E.coli* JM109 strain in the cultivation condition of 30℃ and 180r/min. The infectivity of the full-length SHIV-KB9 in human peripheral blood mononuclear cells (PBMC), cynomolgus and rhesus monkey PBMC was characterized by polymerase chain reaction (PCR), reverse transcription PCR (RT-PCR) and Simian immunodeficiency virus (SIV) p27 gag antigen titration assay.

Key words: SHIV-KB9; Full-genome; Infection; PBMC

Introduction

Human immunodeficiency virus (HIV) is the retrovirus that infects cells of immune system and destroys or impairs their function gradually, leading to "immune deficiency". People with advanced HIV infection are vulnerable to infections or malignancies that are called "opportunistic" because they take advantage of the opportunity offered by a weakened immune system. The symptom complex associated with acquired deficiency of the cellular immune system was called "acquired immunodeficiency syndr-

收稿日期：2003-12-15，修回日期：2003-12-25
* 基金项目：美国国立健康研究所CIPRA资助（U19 AI051915）
作者简介：吴颖运（1975-），女，博士生，研究方向为分子病毒学。
** 通讯作者。Tel: 010-63166184, E-mail: yshao@public3.bta.net.cn
Received: 2003-12-15, Accepted: 2003-12-25
* Foundation item: CIPRA from National Institutes of Health of USA (U19 AI051915)
Author: WU Ying-yun (1975-), female, Ph.D.student, major in molecular virology
** Corresponding author. Tel: 010-63166184, E-mail: yshao@public3.bta.net.cn

ome (AIDS)[1].

Approximately 16,000 individuals become HIV infections worldwide daily and most of them occur in developing countries. The number of individuals diagnosed with HIV in China has risen dramatically in the last ten years. UNAIDS has suggested that China could have 10 million HIV infected people by 2010 if no proper measures are taken [1, 2].

To prevent the aggressive epidemic disease in China, many HIV candidate vaccines have been developed which call for suitable animal model to evalute their officacy and safety. Chimpanzees are the only know primote other than human that could be infected by HIV, but those infections seldom develop into AIDS. Besides, chimpanzees are endangered species and are expensive to obtain and house [3]. As an alternative, SIV and macaques have been used as animal models in AIDS research. Rhesus monkeys and cynomolgus monkeys infected with SIVmac could produce high titers of virus and eventually develope AIDS-like syndromes [4]. However, SIV/ monkey system could not be used directly in evaluation of the HIV-1 vaccine efficacy because of the genetic, antigenic and immunogenic differences between SIV and HIV envelopes.

To overcome these difficulties, a series of recombinant chimeric SHIVs were constructed in several laboratories[5~8]. The *tat*, *rev* and *env* genes of pathogenic clone SIVmac 239 were replaced by the corresponding region of different HIV-1 isolates to generate recombinant SHIV chimeras expressing HIV -1 envelope glycoprotein. These SHIVs containing *env* gene of HIV-1 were widely used as challenge viruses to evaluate the efficacy of anti-HIV-1 vaccine candidates targeting Env proteins in vaccinated monkeys[9~12].

SHIV could also infect rhesus monkey by different transmission routes-----intravaginal, intravenous, intrarectal and oral, which is similar to natural HIV-1 infection of humans. This provides a useful tool to examine the influence of envelope on HIV-1 pathogenesis [13, 14].

In China, researchers have made efforts to develop candidate vaccines, which need develope a suitable SHIV/ macaque animal model to evaluate their efficacy and strategy against HIV transmission. However, there is no paper concerning about the *in vitro* infection and pathogenesis of SHIV-KB9 in Chinese origin rhesus and cynomolgus PBMC.

Usually it was hard to keep full-genome SHIV-KB9 in one plasmid vector for the large size of lentivirus complete genome and highly homology of long terminal repeat sequence of viral genome. The generation of stable full-genome SHIV-KB9 clone will greatly enhance transfection and infection efficiency in cells, and standardize *in vivo* monkey experiment as challenge virus. In addition, the replication assay of SHIV-KB9 in human, Chinese origin cynomolgus and rhesus monkey PBMCs, will make the foundation in evaluation of HIV candidate vaccine in Chinese monkey animal model.

1 Materials and methods

1.1 Construction of full-length SHIV-KB9 clone and its growth condition

Two constructs, SHIV-3′-KB9 and SHIV-5′-KB9, represent 3′ and 5′ parts of SHIV-KB9 viral genome respectively. The 4.0 kb fragment was extracted from SHIV-3′-KB9 digested with *Sph* I / *Not* I, and then ligated with SHIV-5′-KB9 previously treated with the same enzymes. The ligation products of SHIV-KB9 were transformed to JM109 competent bacteria strain and incubated at 30℃ with 180 r/min to ensure the stability of the recombinant proviral DNA in vector. The miniprep plamids of full-genome SHIV-KB9 were characterized with multiple restriction enzymes.

1.2 Transfection of 293T cells with SHIV proviral DNA

In a level 3 biosafety (P3) laboratory, the SHIV-KB9 clone was transfected into 40%~50% confluence 293T cells, which were grown in 10% FBS DMEM (GIBCO) in T-25 flask by using calcium phosphate coprecipitation methods(Promega). The culture supernatants were collected post-transfection 48 hours and filtered with 0.45 μm filter to remove cells. The cell-free viral stock was stored at -80℃ until PBMC infection.

1.3 Preparation of human and monkey PBMC

Human whole blood samples were collected from

healthy volunteers in sterile anticoagulated tube and separated by density gradient centrifugation with Ficoll-hypaque. Monkey blood samples were collected from three healthy adult cynomolgus monkeys and three rhesus monkeys individually. 1.0×10^7 PBMC was isolated from 50 mL human whole blood and $0.5\sim1.0\times10^7$ PBMC was obtained from 6~10 mL monkey blood. The separated PBMCs were washed twice in 1×PBS and washed once in RPMI-1640 with 5% FCS, then cultivated in 20% FCS RPMI-1640 containing 5 μg/mL PHA at 37℃ in 5% CO_2 incubator.

1.4 Infection of human and monkey PBMC with SHIV viral stock

After the stimulation of PHA for three days, PBMC was infected by cell-free SHIV-KB9 viral stock with the p27 titer of 38 ng/mL. Then PBMC cultured in 20% FCS RPMI-1640 containing 25 U IL-2 was washed and resuspended in fresh medium every three days. At post-infection 6, 9, 12 and 15 days, the supernatants were harvested for p27 titration.

1.5 PCR detection of SHIV-KB9 in rhesus monkey PBMC

At post-infection 6, 9, 12 and 15 days, rhesus monkey PBMC were harvested and the whole genomic DNA was extracted with the QIAamp Blood Mini Kit (Qiagen). Specific 477 bp segment in SHIV-KB9 conserved *gag* gene was amplified with forward primer CAG AGA CAT CTA GTG GTG GAA ACA GGA AC ($SIV_{mac}239$ 1360nt-1389nt) and reverse primer AAT GTT GCC TAC TGG TAT GGG GTT TTG TTG ($SIV_{mac}239$ 1808nt-1837nt).

1.6 RT PCR of SHIV-KB9 in human, rhesus and cynomolgus monkey PBMC

At post-infection day 9 of SHIV-KB9, the supernatants from human, rhesus monkey and cynomolgus monkey PBMCs were collected respectively. RNA was extracted from 140 μL cell free supernatants using the QIAamp Viral RNA Mini Kit (Qiagen). To prevent amplification of genomic DNA, primers were designed to hybridize to different exons with forward primer GCA GAG GAG GAA ATT ACC CAG and reverse primer CAA TTT TAC CCA GGC ATT TAA TGTT. The first strand of cDNA was synthesized with the following reactions:10 × PCR buffer (NEB) 2 μL, 10 mmol/L dNTPs 1 μL, 20 μmol/L reverse primer 1.5 μL, 40 U/μL RNase inhibitor (Takara) 0.5 μL, 200U/μL M-MuLV RT (NEB) 0.5 μL, RNA template 10uL and DEPC ddwater 4.5μL. The PCR was performed as follows: 37℃ for 60 min, 95℃ for 5 min and 4℃ for 10 min. Two microliters of reverse-transcription products were used as template to amplify specific fragment of SIV *gag* region. The PCR was run as follows: initial denaturation at 94℃ for 2 min, followed by 30 cycles of 94℃ for 30 s, 56℃ for 30 s and 72℃ for 1 min.

1.7 Analysis of SHIV-KB9 infectivity in human, cynomolgus and rhesus monkeys' PBMC

Human, cynomolgus and rhesus monkey PBMC supernatants were harvested every three days after infection with subsequent addition of fresh 20% FCS RPMI-1640 medium. Samples at day 3, day 6, day 9, day 12 and day 15 were collected and titrated by SIV p27Gag antigen titration (Coulter). SHIV-KB9 viral growth kinetics in human, rhesus and cynomolgous monkey PBMC were monitored according to the titration data.

2 Results

2.1 Construction of full-genome SHIV-KB9

SHIV-KB9 (clone of pathogenic SHIV-89.6P) , which bears with HIV-1 *env* and associated auxiliary genes *tat*, *vpu* and *rev* in the backbone of SIVmac239, had already been serial passaged *in vivo* in rhesus monkeys [15]. Original SHIV-KB9 clone was divided into two parts representing the 3′ and 5′ halves of the viral genome respectively. More than ten full-genome clones of SHIV-KB9 were constructed by ligating the two parts (Fig1).

2.2 The stability of full-length SHIV-KB9

Because of the large size of lentiviruses complete genomes and the highly similar sequence in LTR, it was hard to maintain full-length SHIV genome stable in one plasmid vector. The generated complete genomes SHIV-KB9 provial DNA was maintained stable by using JM109 bacteria strain, lower temperature to 30℃ and lower rotation speed at to 180 r/min cultivation condition. The 37℃ and higher speed rotation had been tried many times, which was commonly used in bacteria cultivation, but none

full-genome SHIV clone had been obtained. Full-length SHIV-KB9 with size of 13141 bp was confirmed grossly intact by multiple restriction mapping (Fig. 2).

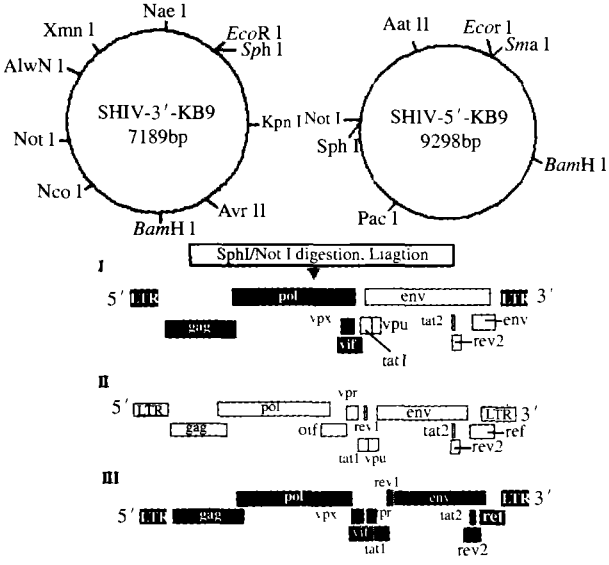

Fig. 1 Construction of full-genome SHIV-KB9 and genomic structure of HIV-1, SIVmac and SHIV-KB9

I. SHIV-KB9 II. HIV-189.6 III. SIVmac239

(■fragment derived from SIV □fragment from HIV-1)

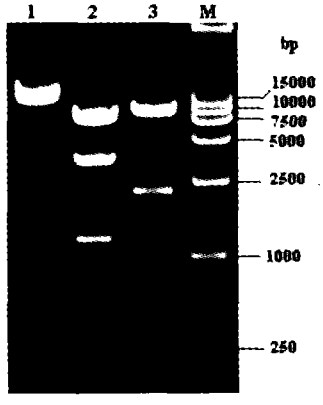

Fig. 2 Restriction ananlysis of recombinant full-genome SHIV-KB9 clone

1. *Sph* I: 13141bp; 2, *Kpn* I: 6657bp, 3516bp, 1207bp, 823bp, 495bp and 443bp; 3. *Bgl* II: 8497bp, 2240bp, 1094bp and 565bp; M, DL15, 000 Mark (Takara)

2.3 PCR of SHIV-KB9 in rhesus monkey PBMC

After the rhesus monkey PBMC infected with SHIV-KB9 viral stock, the specific 477 bp fragment in conserved *gag* region was amplified from rhesus monkey's genomic DNA collected at different days. The result showed that SHIV-KB9 had already integrated into host genomes successfully after the infection (Fig 3).

2.4 RT PCR assay of SHIV-KB9 in human, rhesus and cynomolgus monkey PBMC

At day 9 post-infection, 91 bp fragment within SIV *gag* region was amplified by RT-PCR from the supernatants of infected human, rhesus monkey and cynomolgus monkey PBMC. This result showed that SHIV-KB9 could form full-length *gag-pol* transcript in human, rhesus monkey and cynomolgus monkey PBMC (Fig. 4).

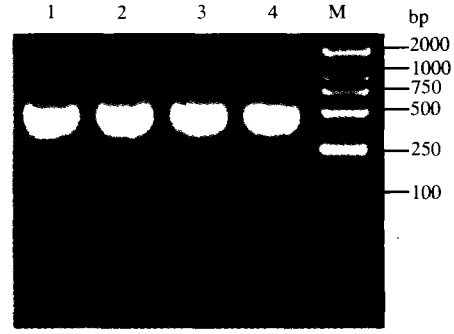

Fig. 3 PCR analysis of the *gag* region of SHIV-KB9 from infected rhesus monkeys' PBMC

1, Rhesus monkey PBMC post-infection day 6; 2, day 9; 3, day 12; 4, day 15; M, DL2000 Mark.

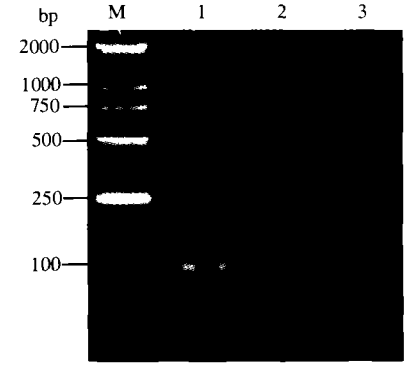

Fig. 4 RT-PCR amplification of 91 bp fragment in full-genome SHIV-KB9 *gag* region

1, Human PBMC supernatants post-infection day 9; 2, Rhesus monkey PBMC post-infection supernatants day 9; 3, Cynomolgus monkey PBMC supernatants post-infection day 9.

2.5 Replication of SHIV-KB9 in human, Chinese cynomolgus and rhesus monkeys PBMC

The replication kinetic of SHI-KB9 was examed every three days in human, Chinese cynomolgus and

rhesus monkeys PBMC (Fig 5). SHIV-KB9 replicated to high titration in all different origin donors' PBMC and usually reached its peak titer at day 9 post-infection. Infected PBMC could produced p27 core antigen titer continuously for 2 to 3 weeks. The difference of replication kinetic among different original donors suggested that the infection and replication of SHIV-KB9 was greatly affected by the immune system of individual host.

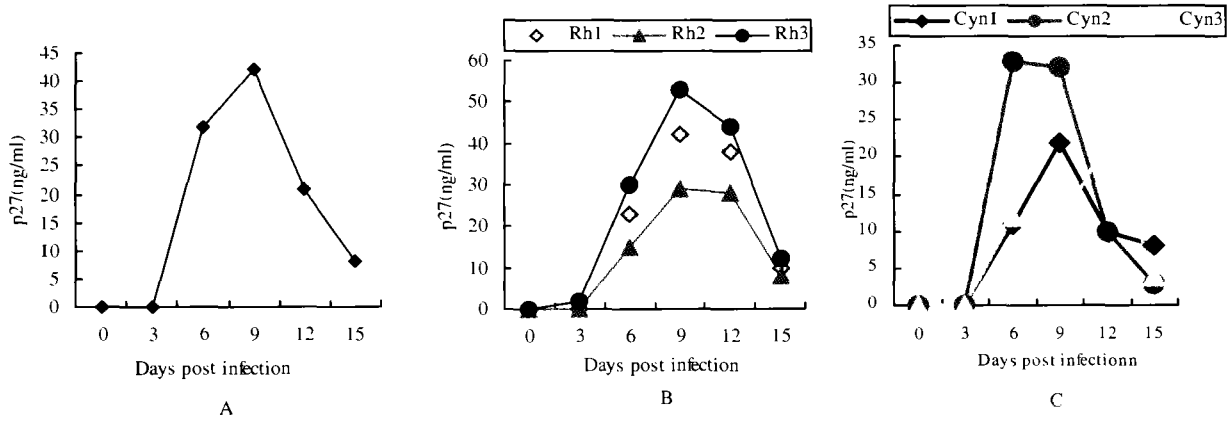

Fig. 5 A. Replication kinetics of SHIV-KB9 in human PBMCs; B. Replication in Chinese rhesus monkey PBMCs; C. Replication in Chinese cynomolgus monkey PBMCs.

3 Discussion

Original SHIV-KB9 clone was divided into two fragments representing the 3′ and 5′ parts of viral genome for safety consideration. However, it was time-consuming to propagate two parts plasmids in large scale, then excise and ligate them to form full-genome SHIV proviral DNA each time before transfection. It is also difficult to harvest infectious SHIV viral stock from transfected 293T cells supernatants for the complicated digestion and ligation procedures.

We failed twice in human PBMC infection experiments with transfection supernants collected from 293T cells, which was transfected with ligation products. Although the p27 titer from 293T cell supernants was high, the viral stock couldn't infect human and rhesus PBMC. To improve the efficiency of transfection and infection, and also to standardize future animal experimental protocol, it is useful to construct stable and infectious full-genome SHIV-KB9 clone. Because of the large size of virus genome and highly similar sequence of LTR, full length clone has great tendency for rearrangements and deletions in one vector, it was not easy to keep full-genome in vector. We failed many times in maintaining the stability of full-genome SHIV and never got any intact clone under 37℃ and high rotation speed cultivation condition. Using JM109 bacteria strain as host and controlled growth condition, this problem has been solved successfully.

In addition, not all full-genome clones maintained the infectivity and pathogenesis because anything mutation or deletion in SHIV genome may cause fatal deficiency and the losing of biological activity. More than ten full-genome SHIV- KB9 clones were constructed with correct restriction mapping under the controlled growth condition, However most of them didn't displayed lost biological function and infectivity (data not shown).

A full-genome SHIV-KB9 clone was constructed successfully, which could be maintained stable under special growth conditions. *In vitro* its infectivity was confirmed in human PBMC donor and three Chinese rhesus, cynomolgus monkey PBMCs. All infected PBMC displayed high titration suggesting the infectivity and pathogenesis of SHIV- KB9 in Chinese rhesus and cynomolgus monkeys.

SHIV-KB9 has been widely used as challenge virus in SHIV/macaque animal model. In the near future, SHIV-KB9 will also play an important role in Chinese SHIV/monkey animal model to evaluate the

efficacy of China's HIV condidate vaccines and related vaccination protection protocols against HIV infection.

Reference

[1] UNAIDS. AIDS epidemic update: 2003. UNAIDS Web Site. Available at: http://www.unaids.org/publications/

[2] Portsmouth S, Stebbing J, Keyi X, *et al*. HIV and AIDS in the People's Republic of China: a collaborative review[J]. Int J STD AIDS. 2003 Nov; 14(11): 757-61.

[3] Smaglik P. AIDS vaccine researchers turn from chimps to monkeys [J]. The Scientist, 1999, 13(16): 7-9.

[4] Veazey R S, Lifson J D, Pandrea I, *et al*. Simian immunodeficiency virus infection in neonatal macaques [J]. J Virol. 2003 Aug; 77(16): 8783-92.

[5] Li J Lord C I, Haseltine W, *et al*. Infection of cynomolgus monkeys with a chimeric HIV-1/SIVmac virus that expresses the HIV-1 envelope glycoproteins[J]. J Acquir Immune Defic Syndr, 1992, 5(7): 639-646.

[6] Luciw P A, Pratt-Lowe E, Shaw K E, *et al*. Persistent infection of rhesus macaques with T-cell-line-tropic and macrophage-tropic clones of simian/human immunodeficiency viruses (SHIV) [J]. Proc Natl Acad Sci U S A, 1995, 92(16): 7490-7494

[7] Sakuragi S, Shibata R, Mukai R, *et al*. Infection of macaque monkeys with a chimeric human and simian immunodeficiency virus[J]. J Virol, 1992, 73(Pt 11): 2983-2987.

[8] Joag S V, Li Z, Foresman L, *et al*. Chimeric simian/human immunodeficiency virus that causes progressive loss of $CD4^+$ T cells and AIDS in pig-tailed macaques[J]. J Virol, 1996, 70(5): 3189-3197.

[9] Ruprecht R M, Hofmann-Lehmann R, Smith-Franklin B A, *et al*. Protection of neonatal macaques against experimental SHIV infection by human neutralizing monoclonal antibodies[J]. Transfus Clin Biol, 2001, 8(4) 350-358.

[10] Ui M, Kuwata T, Igarashi T, *et al*. Protection of macaques against a SHIV with a homologous HIV-1 Env and a pathogenic SHIV-89.6P with a heterologous Env by vaccination with multiple gene-deleted SHIVs[J]. Virology, 1999, 265(2): 252-263.

[11] Joag S V, Li Z, Foresman L, *et al*. Characterization of the pathogenic KU-SHIV model of Acquired Immunodeficiency Syndrome in macaques [J]. AIDS Res Hum Retroviruses, 1997, 13(8): 635-645.

[12] Ruprecht R M, Ferrantelli F, Kitabwalla M, *et al*. Antibody protection: passive immunization of neonates against oral AIDS virus challenge [J]. Vaccine, 2003, 21(24): 3370-3373.

[13] Bogers W M, Cheng-Mayer C, Montelaro R C. Developments in preclinical AIDS vaccine efficacy models [J]. AIDS, 2000, 14 (Suppl 3): S141-512.

[14] Pauza C D, Lu X, *et al*. Rhesus macaques that become systemically infected with pathogenic SHIV 89.6-PD after intravenous, rectal, or vaginal inoculation and fail to make an antiviral antibody response rapidly develop AIDS [J]. J Acquir Immune Defic Syndr Hum Retrovirol, 1998, 19(1): 6-18.

[15] Etemad-Moghadam B, Sun Y, Nicholson E K, *et al*. Envelope glycoprotein determinants of increased fusogenicity in a pathogenic Simian-Human Immunodeficiency Virus (SHIV-KB9) passaged in vivo[J]. J Virol, 2000, 74(9): 4433-4440