

Acute Infection of Chinese Macaques by a CCR5-Tropic SHIV Carrying a Primary HIV-1 Subtype B' Envelope

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Abstract: The increasing prevalence of HIV-1 subtype B in China and Southeast Asia calls for efforts to develop a relevant animal model to study viral transmission and pathogenesis. Because there are significant differences between subtype B HIV-1 and other chimeric simian/human immunodeficiency virus (SHIVs) in the *env* gene, a novel SHIV, designated SHIVB_{WHU}, was generated by replacing counterparts of SHIVSF33 with *tat/rev/vpu/env* genes derived from a primary, CCR5-tropic, subtype B' HIV-1 strain of a Chinese patient. SHIVB_{WHU} was able to replicate in rhesus peripheral blood mononuclear cells and used CCR5 as its major coreceptor. Moreover, after serial passages in Chinese macaques, the *in vivo* infectivity of SHIVB_{WHU} was enhanced, yet no significant sequence changes were found in viral envelopes, and the virus did not change its CCR5-tropism. CD4⁺ T-cell loss, however, was found in the intraepithelial lymphocytes of small intestines of infected macaques. Our findings have implications in understanding the early pathogenesis of SHIVB_{WHU} in Chinese macaques.

Key Words: acute infection, animal model, Chinese macaques, SHIV, subtype B'

(*J Acquir Immune Defic Syndr* 2009;00:000–000)

INTRODUCTION

Human immunodeficiency virus type one (HIV-1) epidemic in China is largely driven by several high-risk factors.¹ One of the risk factors is paid blood donation (PBD), which was initially identified in Henan province, where many poor farmers were infected through illegal blood collection practices without adequate screening and sterilization procedures.^{2,3} Many of the infections occurred when contaminated blood cells were transfused back to donors once the plasma had been removed from the pooled blood. Over the past years, such activities, which peaked between 1992 and 1996, have caused hundreds of thousands of new infections among paid blood donors in rural areas in Henan and neighboring provinces including Anhui, Hubei, Hebei, Shaanxi, and Shanxi,^{2,4} creating the tragic “AIDS villages”^{5,6} and attracting tremendous public attention.^{7,8} Although illegal PBD has been banned by the Chinese government, the increasing prevalence of HIV-1 subtype B' through other risk behaviors including unprotected sexual contacts still calls for efforts to fight the epidemic.

To fulfill the goal of controlling HIV-1 dissemination from high-risk groups into general populations,^{9,10} there is a need to understand the viral transmission and to develop an effective preventive means. Because many preventive means cannot be tested in humans, it is desirable to develop a relevant animal model. Therefore, a lot of efforts have been put on the development of the chimeric simian/human immunodeficiency virus (SHIV).^{11–13}

Most of currently available SHIVs utilize envelope genes derived from HIV-1 subtype B strains, either from laboratory-adapted, syncytium-inducing, T-tropic viruses (HIV-1_{HXB2} and HIV-1_{NL43}) or from primary, nonsyncytium-inducing, M-tropic (HIV-1_{SF162}), syncytium-inducing T-tropic (HIV-1_{SF33}) and dual-tropic (HIV-1_{89.6} and HIV-1_{DH12}) isolates.^{14,15} In addition, a few nonsubtype B SHIVs were generated for subtype C and E including the SHIV_{CHN19} that we previously generated to study the major HIV-1 epidemic caused by subtype C and CB recombinant viruses in China.^{16–18} Till now, however, no SHIV has been generated based on the envelope of subtype B' HIV-1, another major epidemic strain in China and Southeast Asia. Moreover, it remains unknown whether or not a SHIV generated from a freshly isolated human HIV-1 would be pathogenic *in vivo* during acute phase of infection. Here, we report the construction and characterization of a new SHIV_{B'WHU} both *in vitro* and *in vivo*.

Received for publication August 5, 2009; accepted November 18, 2009.

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Supported by the National Basic Research Program of China (the 973 project 2006CB504208), the National 11th 5-year project 2008ZX10001-011, Hong Kong research grant council (HK-RGC762208 and HK-RGC762209 to ZC), and the University Development Fund of the University of Hong Kong to its AIDS Institute.

The authors declare no financial or commercial conflict of interest.

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Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.jaids.com).

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MATERIAL AND METHODS

Amplification of HIV-1 *tat/rev/vpu/env* Genes

The genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) of an HIV-1-infected PBD (02HN_{SMX2}).² The HIV-1 *tat/rev/vpu/env* fragment was amplified from genomic DNA by a nested polymerase chain reaction (PCR) using the EXPAND high-fidelity DNA polymerase according to the manufacturer's specifications (Boehringer-Mannheim, IN). The outer primer pair is 4759F1 (5'-GTT TTT CAG AAT CTG CTA TAA GAA ATG CC-3'), BU5 (B) (5'-CTC TAG TTA CCA GAG TCA CAC AAC AGA CGG GCA CAC AC-3') and the inner primer pair is VprA (5'-TGCC *GAATTCGCATGC* TA TAG A TAG AGG AGA GCA AGA AAT GGA G -3') and EnvB (5'-TGCC *CTCGAG* CT TAT AGT AAA GCC CTT TCG AGG-3'), where the introduced stop codons are underlined and the restriction enzyme sites (*EcoRI*, *SphI*, and *XhoI*) are italicized. Products from the second-round amplifications were purified from agarose gels using QIAquick gel extraction kit (Qiagen, Valencia, CA).

Construction of SHIV_{B'WHU}

The *env* sequence was subjected to the phylogenetic analysis as previously described.¹⁶ PVP-1 plasmid contains the 5'-half SIV_{mac239} genome from the 5' long terminal repeat, a generous gift of Dr Paul Luciw. 3' half vpu⁺-B'_{WHU} plasmid (3'-p02HN_{SMX2}-B'_{WHU}) contains the 3'-half of SHIV_{B'WHU} genome. The 3'-p02HN_{SMX2}-B'_{WHU} plasmid was made by replacing counterparts of SHIV_{SF33} (a generous gift of Dr Cecilia Cheng-Mayer) with newly obtained subtype B' *tat/rev/vpu/env* genes. To make a replication-competent recombinant SHIV_{B'WHU}, linear 5'-PVP-1 and 3'-p02HN_{SMX2}-B'_{WHU} plasmids were co-transfected into 293T cells using Effectene Transfection Reagent (Qiagen, Valencia, CA). Forty-eight hours later, 1×10^7 CEM \times 174-5.25M7 cells in DMEM media were placed into the 60 mm dishes containing the transfected 293T cells and supernatant. The culture supernatants were harvested for p27 measurement and used to infect macaque PBMCs. Some transfected 293T cells were also subjected to the cell fusion assay to test the expression and phenotype of Env as we previously described.¹⁶

Genetic Analysis of Amplified HIV-1 Genes

Full-length *tat/rev/vpu/env* amplicon was cloned into the expression vector pcDNA-I/Amp (Invitrogen, Carlsbad, CA) and was subsequently sequenced. The full *env* sequence was subjected to phylogenetic analysis.^{1,2} The phylogenetic tree was generated using the neighbor-joining method implemented in the ClustalX1.8 program. The tree was plotted using the TreeView program.¹⁹ The SimPlot and BootScan analysis was used to determine the possible intersubtype recombination.²⁰

Replication of SHIV_{B'WHU} in Chinese Macaque PBMCs

Macaque blood was obtained from healthy adult Chinese rhesus monkeys that were provided by Wuhan University Laboratory Animal Center. PBMCs were separated from the

whole blood by centrifugation through Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) density gradients. To test the infectivity of newly generated SHIV_{B'WHU} strain, 1×10^7 stimulated macaque PBMCs were infected by cell-free virus with an equal inoculum (p27 titer: 0.5 ng/mL). The infected PBMCs were cultured in 10% FBS RPMI-1640 containing 20U IL-2. On day 5, 10 and 15 postinfection, the viral supernatants were harvested for p27 measurement. The p27 level was measured using a kit purchased from ZeptoMetrix Corporation (Buffalo, New York, NY).

FACS ANALYSIS

The number of peripheral CD4⁺ and CD8⁺ lymphocytes in SHIV-infected animals was determined by fluorescence-activated cell sorter using fluorochrome-conjugated monoclonal antibodies as described previously.¹⁶

Inoculation of Chinese Macaques With SHIV_{B'WHU}

Four juvenile Chinese rhesus macaques were infected with SHIV_{B'WHU} in this study. A dose of 1.1 μ g p27 in a volume of 15 mL of cell-free viral supernatant was infused intravenously via the femoral vein into a Chinese macaque (P1-#0002). For serial passage, 10 mL of heparinized whole blood and 5 mL of bone marrow collected from the P1-#0002 macaque at 2 weeks postinoculation were inoculated intravenously, also via the femoral vein, into another healthy Chinese macaque (P2-#0296). Subsequent passages for P3 (P3-#1032) and P4 (P4-#1050) animals were carried out in the same manner as for P2-#0296 animal. Our experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the Laboratory Animal Center in Wuhan University Laboratory Animal Center, where the experimental animals were housed.

Viral Load Measurement

The level of plasma viremia and viral RNA and proviral DNA loads in tissues was measured by a quantitative real-time PCR method as described previously.^{16,17} A molecular beacon-based real-time PCR assay was used to quantify the copy number of SHIV_{B'WHU} in plasma using the ABI 7700 PRISM spectrofluorometric thermal cycler (Applied Biosystems, Foster City, CA). The limit of detection for plasma viral load was approximately 400 RNA copies per milliliter after the adjustment of the dilution factors.

Immunohistochemistry

Tissue sections from formalin-fixed paraffin-embedded small intestines (SIs) (jejunum) were stained with a mouse anti-human CD4 monoclonal antibody (NCL-CD4-1F6; Novacastra Laboratories, Ltd, London, United Kingdom) using the DakoCytomation-Labelled Streptavidin-Biotin2 System, Horseradish Peroxidase (LSAB2 System, HRP, DAKO Corporation, Carpinteria, CA) and AEC Substrate-Chromogen solution (DAKO Corporation) as previously described.^{21,22}

Determination of Coreceptor Usage

The coreceptor usage of the postpassage SHIV_{B'WHU} was determined by using GHOST CD4 cells expressing each of the following proteins including CCR1, CCR2b, CCR3, CCR4, CXCR4, CCR5, CCR8, Bob, Bonzo, and V28. Viral isolates were used to infect each of the cell types in duplicate in 96-well plates at a multiplicity of infection of 1.0. After incubation at 37°C for 2 hours, the cultures were washed 3 times with phosphate-buffered saline and maintained in the culture medium. The cells were photographed by fluorescent microscopy to detect GFP expression 24–48 hours postinoculation.

RESULTS

Sequence Analysis of SHIV_{B'WHU} *env* Gene

To generate a subtype B' SHIV, we amplified the *tat/rev/vpu/env* fragments of HIV-1 subtype B'WHU from the proviral genome of a patient's PBMCs (02HN_{SMX2}) by a nested PCR. Sequencing results indicated that the open-reading frames of *tat/rev/vpu/env* genes were complete without any frame shift. Because viral envelope determines viral genotype and phenotype,²³ a phylogenetic analysis was conducted to determine the viral genotype as previously described.¹⁶ As depicted in the neighbor-joining tree (Fig. 1), the SHIV_{B'WHU} *env* clustered tightly with subtype B' strains identified either in Henan (02HN_{SMX2}, 02HN_{SC11}, and 02HN_{SC4}), Yunnan (B'YN.RL42) of China or in other Southeast Asian countries such as Thailand (subtype B'.TH.93TH067) and Myanmar (subtype B'.MM.99.mSTD101). In contrast, *env* sequences from western countries, including those from the United States, are divergent from subtype B' virus (Fig. 1).^{2,24} Furthermore, the SimPlot and BootScan analysis showed that the *env* of SHIV_{B'WHU} is a pure B' without cross over or recombination with viruses of other subtypes, including subtype C/B' viruses currently circulating in China.^{1,16} When compared with *env* genes of other SHIV strains including SHIV_{89.6}, SHIV_{SF33}, SHIV_{SF162}, SHIV_{KU2}, SHIV_{1157ipd3N4}, SHIV_{CHN19}, SHIV_{MJ4}, and SHIV_{E-CAR} (Fig. 1),^{18,25} SHIV_{B'WHU} *env* is the only unique one representing the subtype B' viral cluster. In addition, there were significant differences between SHIV_{B'WHU} and other SHIVs especially in variable regions (V1, V2, V3, V4, and V5) based on the aligned amino acid sequences (See **Figure, Supplemental Digital Content 1**, <http://links.lww.com/QAI/A28>). The amino acid similarity between SHIV_{B'WHU} and each of these SHIV strains as mentioned above is 82%, 83%, 85%, 83%, 77%, 77%, 73%, and 75%, respectively.

Serial Passages of SHIV_{B'WHU} in Chinese Macaques

We first determined that SHIV_{B'WHU} used CCR5 as its coreceptor and was able to replicate in rhesus PBMCs (data not shown). To enhance the infectivity of SHIV_{B'WHU}, in vivo serial passage of SHIV_{B'WHU} was performed in 4 naive Chinese rhesus macaques.^{16,26} Cell-free SHIV_{B'WHU} supernatant was inoculated intravenously into a healthy Chinese rhesus monkey (P1-#0002). Fourteen days after the inoculation, 10 mL of heparinized blood and 5 mL of bone marrow from this animal

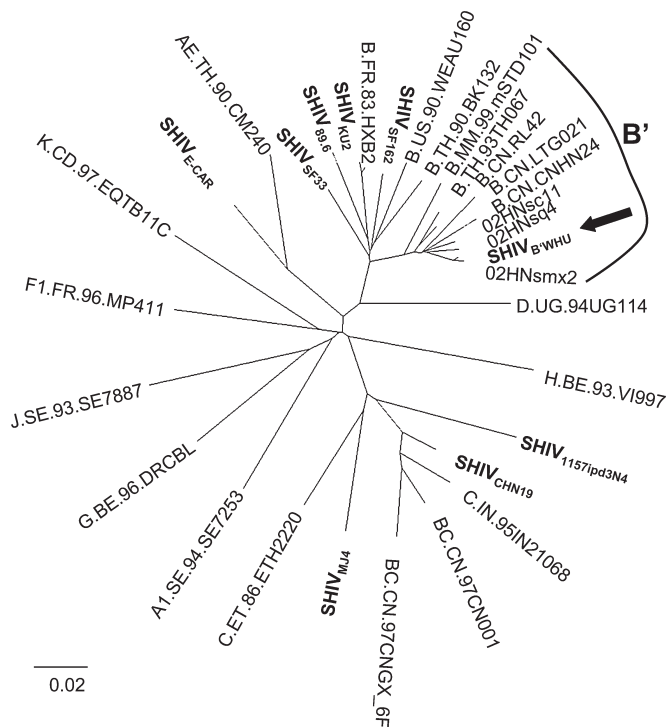


FIGURE 1. Phylogenetic analysis of the full-length *env* gene of SHIV_{B'WHU}. SHIV_{B'WHU} is indicated by the arrow. The reference sequences were obtained from the HIV sequence database in GenBank. The tree was constructed by using full-length *env* nucleotide sequences by a neighbor-joining method as previously described.^{1,2} The length of branches is additive and represents genetic distance between pairs of viruses. The solid scale bar indicates a genetic distance of 0.02. The reference SHIV strains are shown in bold.

was inoculated intravenously into a second naive animal (P2-#0296). Such in vivo passage was carried out sequentially into the third and the fourth naive rhesus monkeys (P3-#1032 and P4-#1050), respectively. Unexpectedly, there was no detectable viremia in P1-#0002 during the 14 days of infection (Fig. 2). P2 animal (P2-#0296), however, developed productive infection as indicated by a burst of viremia (10^6 copies of viral RNA per mL of plasma) 14 days after the blood-bone marrow transfusion. Further in vivo passages of SHIV_{B'WHU} have improved the viral infectivity because the burst of viral replication was detected earlier on day 12 with viral loads ranging 10^6 to 10^7 copies of viral RNA per milliliter of plasma (Fig. 2). During these 14 days of acute infection, there was no significant change in peripheral CD4⁺ and CD8⁺ T-cell counts in all 4 infected Chinese rhesus macaques.

Quantification of Proviral DNA and Viral RNA in Tissues

Because SHIV_{B'WHU} is a R5-tropic virus, we were curious to understand viral infection and pathology in the guts during the acute phase of infections, which has not been previously studied with any SHIVs before in vivo adaptation. Therefore, all animals were sacrificed 2 weeks postviral inoculation. Proviral DNA and viral RNA loads in tissues were

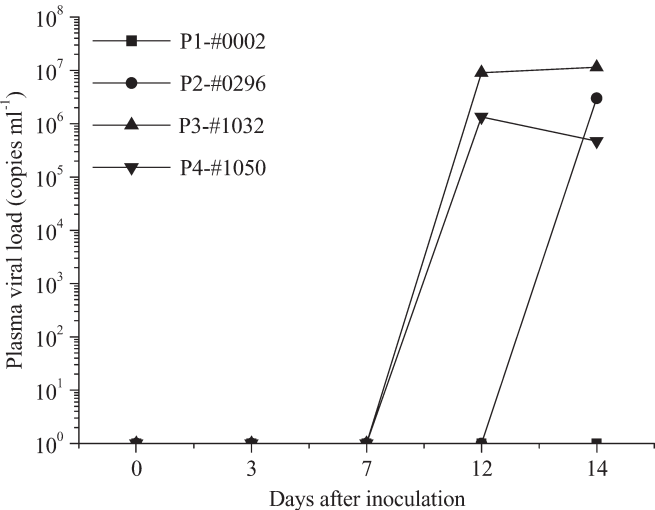


FIGURE 2. Characterization of plasma viral load in infected animals. The viral load of infected animals was measured using a real-time PCR assay. The Y axis represents viral RNA copies per milliliter of plasma. The X axis indicates the time post-inoculation.

determined by a quantitative real-time PCR assay as previously described.^{17,27} In this analysis, SI, axillary lymph node (ALN) and mesenteric lymph node were examined in all animals (Fig. 3). Interestingly, despite the absence of detectable viral RNA in peripheral blood of P1-#0002, there was significant level of viral RNA load and proviral DNA load in ALN of this animal (Fig. 3A). In P2-#0296 monkey, accompanying the burst of viremia on day 14 post transfusion, proviral DNA and viral RNA were mainly detected in SIs in addition to low levels in ALN. In P3-#1032 and P4-#1050 monkeys, with the earlier burst of viremia on day 12 postinoculation, the viral RNA load and proviral DNA load were significantly higher in SIs. Moreover, both proviral DNA

and viral RNA load were significantly higher in mesenteric lymph nodes (Fig. 3B), which belongs to mucosal tissue compartments. It is possible that the enhanced viral infectivity is related to the increasing infection of CD4⁺ T lymphocytes in mucosal tissue compartments. As for the failure of detectable viral load in other tissues, we believed that it was probably due to the limit of our viral load assay because viral genes were readily detected in all tissue compartments tested by nested PCR.

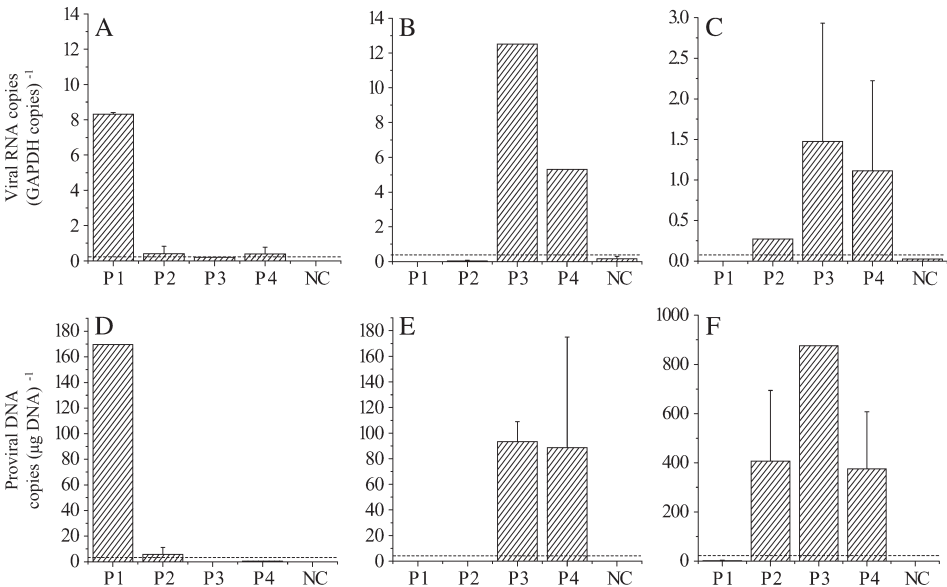
Immunohistochemistry Analysis of CD4⁺ T Cells in SIs

To further determine the pathogenicity of CCR5-tropic SHIV_{B-WHU} during in vivo passages, the cytopathic effect of viral infection was investigated in the SIs. We were unable to analyze the CD4⁺ T cells using the flow cytometry technique due to the lack of equipment at our study site. Immunohistochemistry analysis, however, was performed on formalin-fixed paraffin-embedded SIs to determine the loss of CD4⁺ T cells using a mouse anti-human CD4 monoclonal antibody.²² CD4⁺ T cells were stained red as shown in Fig. 4. For each monkey, we analyzed multiple tissue sections and multiple fields of each tissue section. Compared with uninfected rhesus monkeys, the number of CD4⁺ T cells in SHIV_{B-WHU}-infected monkeys was significantly reduced especially among the intraepithelial lymphocytes (IEL) of SIs. CD4⁺ T cells were detected among the lamina propria lymphocytes but with reduced numbers, and in mucosa-associated lymphocyte aggregates or tissues (Fig. 4).

Unchanged CCR5-Tropism of SHIV_{B-WHU} After Serial Passage

To determine the viral coreceptor usage, viruses were reisolated from P2-P4 passaged monkeys. These viral isolates were used to infect GHOST CD4 cells expressing each of the coreceptors, including CCR1, CCR2b, CCR3, CCR4, CXCR4, CCR5, CCR8, Bob, Bonzo, and V28. These isolates

FIGURE 3. The quantification of viral RNA (top) and proviral DNA (bottom) in different tissues: Axillary LN (A, D), Mesenteric LN (B, E), and SI (C, F). Viral RNA copies (GAPDH copies)⁻¹ and proviral DNA copies (μg DNA)⁻¹ were determined by real-time PCR assays. The error bar indicates the experimental variation, whereas the dot line indicates the cut-off value. Viral *env* genes were amplified by nested PCR for sequence analysis from all tissue compartments as shown here. NC refers to uninfected negative control.



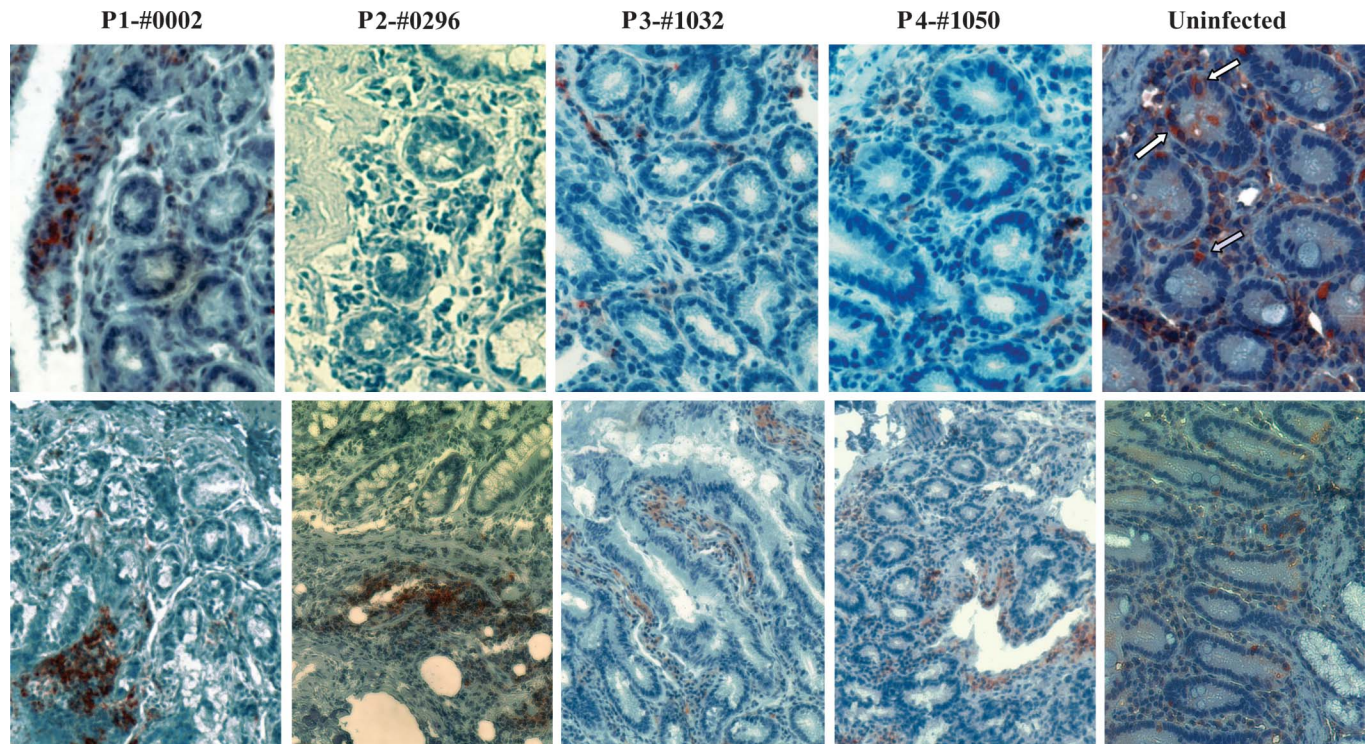


FIGURE 4. Immunohistochemistry analysis of CD4⁺ IEL loss in SIs of infected macaques. CD4⁺ T lymphocytes were detected in SIs of infected and control macaques with a mouse anti-human CD4 monoclonal antibody at 2 weeks postinfection. CD4⁺ cells are in red. The arrows showed the CD4⁺ IELs. At least 5 tissue sections and multiple fields of each section were analyzed for each monkey. Original magnifications: $\times 400$ (top panel) and $\times 200$ (bottom panel).

infected GHOST CD4 Hu-CCR5 cells but not other cells (See **Figure, Supplemental Digital Content 2**, <http://links.lww.com/QAI/A29>), with a pattern similar to that of the parental SHIV_{B'WHU}. Furthermore, there were only low frequent sporadic amino acid substitutions identified in their *env* fragments. These results suggest that, despite in vivo serial passage, SHIV_{B'WHU} has maintained CCR5 specificity with no evidence of altered coreceptor utilization.

DISCUSSION

Over the past years, Asia has become the second most heavily affected continent by HIV/AIDS in the world. Because Asia has the world's largest human population, effective measures must be implemented to control the increasing prevalence of HIV-1 infection. To this end, the development of a relevant animal model to facilitate the testing of various preventive measures (eg vaccine, microbicide, antivirals) remains necessary. HIV-1 subtype B' is a major HIV-1 genotype not only in China but also in other Southeast Asia countries.^{2,28} Because there is a significant amount of sequence variation in *env* genes of subtype B' and western B viruses (See **Figure, Supplemental Digital Content 1**, <http://links.lww.com/QAI/A28>), currently available B SHIVs are not necessarily suitable for studies of subtype B' viral transmission and pathogenesis. For example, recent studies indicated that some subtype B' HIV-1 strains are less susceptible to a few potent neutralizing antibodies derived from patients infected with western B viruses.²⁹ The newly constructed

SHIV_{B'WHU} is therefore particularly useful for studying issues related to subtype B' *env*-mediated infection in a nonhuman primate model.

The infectivity of SHIV_{B'WHU} was enhanced in Chinese macaques after in vivo serial passage. Before in vivo passage, the phenotype and replication kinetics of SHIV_{B'WHU} was determined by in vitro assays. SHIV_{B'WHU} was found to be CCR5-tropic similar to its primary parental HIV-1 isolate (See **Figure A, Supplemental Digital Content 3**, <http://links.lww.com/QAI/A30>). This phenotype is crucial because it represents the dominant type of sexually transmitted HIV-1 strains.^{23,30} Furthermore, SHIV_{B'WHU} was demonstrated to be replication-competent in PBMCs of 2 randomly selected Chinese macaques (See **Figure B, Supplemental Digital Content 3**, <http://links.lww.com/QAI/A30>), which warranted the subsequent in vivo experiments. Although no detectable viremia was found in peripheral blood of P1-#0002 monkey at the acute phase of infection, the subsequent increasing viremia detected in P2-P4 animals suggested that SHIV_{B'WHU} has adapted to grow in vivo and therefore became infectious in Chinese macaques (Fig. 2). In general, the observed pattern of SHIV_{B'WHU} replication in macaques is similar to previous findings of SHIV infection in a new monkey host.¹⁶ Consistently, the improved growth kinetics and the broader tissue tropism of SHIV_{B'WHU} in P2-P4 animals suggested the enhanced viral infectivity in vivo (Figs. 2, 3). The enhanced infectivity, however, did not result in altered CCR5-tropism and therefore, unlike pathogenic X4-tropic and X4R5-tropic SHIV strains which

induce severe CD4⁺ T-cell loss in the periphery,^{23,31} no significant CD4⁺ lymphocytopenia was found in peripheral blood of SHIV_{B*WHU}-infected macaques. In addition, although we see enhanced viral replications in P3 and P4 macaques, we cannot exclude the possibility that the target cells of P1 and P2 are less susceptible to SHIV_{B*WHU} infection.

The rapid in vivo serial passage improved the infectivity of SHIV_{B*WHU} probably by improving viral fitness and extending the involvement of tissue CD4⁺ T lymphocytes in vivo. Previous investigators demonstrated that serial passage of SHIV in macaques resulted in virus with high pathogenicity.^{18,32,33} The pathogenicity was often indicated by high viral load, CD4⁺ T lymphocytopenia, and even simian AIDS. In this study, we found that a freshly made (before adaptation in macaques) CCR5-tropic SHIV was able to deplete CD4⁺ IELs and to less extent the CD4⁺ lamina propria lymphocytes in all 4 macaques tested. This finding suggested that CD4⁺ IELs were probably more susceptible to the acute infection of SHIV_{B*WHU} even without the overtime adaptation of the virus in vivo. However, the number of experimental animals is small in our study. Because similar studies were not conducted for other CCR5-tropic SHIVs, it remains to be determined whether our finding is a general phenomenon for viruses of the same phenotype. Moreover, a more quantitative FACS analysis is needed to determine the quantitative loss of CD4⁺ IEL in SHIV_{B*WHU}-infected animals in future study. Previous studies indicated that some changes were also detected in the IELs population during SIV infection,³⁴ it might be critical to determine the role of IELs in seeding the early transmission and replication of CCR5-tropic HIV-1. Because the viral tropism was not changed in P3 and P4 monkeys, it is therefore possible that the improved infectivity of SHIV_{B*WHU} was not simply due to its phenotypic changes. It is likely that the early involvement of CD4⁺ T lymphocytes in broad tissue compartments also contributed to the rapid burst of viremia especially in P3 and P4 animals (Fig. 3). To confirm the improved infectivity of the virus, 3 additional Chinese macaques were recently inoculated with SHIV_{B*WHU} derived from the P4 animal. All 3 animals have become infected as indicated by peak viremia and subsequent seroconversion. These animals will be used to determine whether or not SHIV_{B*WHU} would lead to persistent infection and subsequent simian AIDS in Chinese macaques. In addition, to build a useful SHIV_{B*WHU}/Chinese macaque model, it would also be necessary to determine the mucosal transmissibility of SHIV_{B*WHU} with enhanced infectivity in future studies. Lastly, because different macaque species may respond to SIV or SHIV infection differently, future studies should determine the pathogenicity and disease course of SHIV_{B*WHU} infection in Indian or pig-tailed macaques.

ACKNOWLEDGMENTS

We thank Drs D. D. Ho and K. Y. Yuen for scientific advice and C. Cheng-Mayer for SHIV₃₃ molecular clone and discussions.

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