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IV. 研究成果の刊行物・別刷

J Med Primatol doi:10.1111/jmp.12084

ORIGINAL ARTICLE

Development of a novel rhesus macaque model with an infectious R5 simian–human immunodeficiency virus encoding HIV-1 CRF08_BC *env*

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Keywords
Chinese rhesus macaques – CRF08_BC – peripheral blood mononuclear cell – R5 – simian–human immunodeficiency virus

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Accepted August 19, 2013.

Abstract

Background The CRF08_BC strain is one of the most predominant circulating Human immunodeficiency virus type 1 (HIV-1) strains in the Chinese pandemic. A simian–human immunodeficiency virus (SHIV) encoding HIV-1 CRF08_BC *env* is highly desirable to evaluate candidate AIDS vaccines in non-human primates.
Methods SHIV-KBQJ-12, which carries the envelope glycoprotein from QJ001, an infectious molecular clone of HIV-1 CRF08_BC, was generated. The replication capacity of SHIV-KBQJ-12 was determined both in human and rhesus macaque (*Macaca mulatta*) peripheral blood mononuclear cells (PBMCs) and in Chinese rhesus macaques.
Results SHIV-KBQJ-12 replicated efficiently in human and macaque PBMCs and displayed a preference for CCR5 as an entry coreceptor. Productive infection of two macaques by intravenous inoculation with SHIV-KBQJ-12 was confirmed.
Conclusions SHIV-KBQJ-12 is an R5-tropic chimeric virus that can establish productive infection both *in vitro* and *in vivo* in Chinese rhesus macaques and will be useful to assess candidate HIV-1 CRF08_BC vaccines in China.

Introduction

By the end of 2011, 0.78 million people (estimates of 0.62 million to 0.94 million) were living with HIV/AIDS in China (http://www.unaids.org.cn). Two Chinese nationwide HIV molecular epidemiology surveys have revealed that the proportion of subtype CRF_BC, including CRF07_BC and CRF08_BC, has increased dramatically, and the CRF_BC strain has become the most prevalent subtype in the Chinese pandemic, accounting for 50.2% of all human immunodeficiency virus type 1 (HIV-1) clades in a recent nationwide HIV molecular epidemiology survey (2001–2003) [1, 2]. Several candidate vaccines against the HIV-1 CRF_BC subtype have been designed to control the Chinese prevalence of HIV more effectively [3, 4]. To accurately evaluate the efficacy and safety of these candidate vaccines, a relevant animal model is needed urgently.

A significant challenge in HIV-1 research is that the only animal susceptible to HIV-1 infection is the chimpanzee (*Pan troglodytes*), which is an endangered species [5]. Accordingly, simian immunodeficiency virus (SIV) infection of rhesus macaques (*Macaca mulatta*) has been used as an alternative animal model

for HIV-1 infection in humans. However, the SIV/macaque model is limited by genetic variation between HIV-1 and SIV. To address this problem, a simian–human immunodeficiency virus (SHIV)/rhesus macaque system was constructed and is now widely used to study the role of HIV-1 genes in transmission and pathogenesis as well as to evaluate the efficacy of candidate vaccines based on HIV-1 envelope glycoproteins, which specify cell tropism and coreceptor usage and are also primary targets of the immune response [6–11]. SHIV clones are usually constructed by replacing the envelope gene region of the SIV pathogenic clone SIVmac239 with the counterpart from HIV-1 [12–17]. In addition, SHIV strains always induce simian AIDS in macaques and mimic HIV disease progression in humans through *in vivo* adaptation and serial *in vivo* passaging [7, 18, 19]. However, the majority of current SHIV strains utilize envelope genes derived from HIV-1 subtype B strains and fail to reflect the genetic diversity of the Chinese HIV epidemic, which is dominated by the HIV-1 subtype CRF_BC. HIV-1 CRF_BC is a new circulating recombinant form of subtype B' (Thailand's variant of subtype B) and subtype C, and thus, the envelope gene of CRF_BC is highly similar to but not identical to that of the clade C virus. To date, only four HIV-1 subtype C envelope-based SHIV chimeras, including SHIV-CHN19, SHIV-MJ4, SHIV-MCGP1.3, and SHIV-1157ipd3N4, and three HIV-1 CRF07_BC envelope-based SHIV chimeras, including SHIV-XJ02170, SHIV-XJDC6431, and SHIV-CN97001, have been reported [7, 11, 20–24]. However, most of the SHIVs are deficient and cannot induce simian AIDS in macaques, with the exception of SHIV-1157ipd3N4. In particular, no SHIV strain encodes the envelope gene of HIV-1 CRF08_BC. Therefore, an SHIV/rhesus macaque infectious model based on HIV-1 CRF08_BC is needed.

One of the most widely used SHIV strains, SHIV-89.6P, encodes HIV-1 subtype B *env* and induces rapid $CD4^+$ lymphocyte depletion and AIDS-like disease in rhesus monkeys after two serial *in vivo* passages [25, 26]. SHIV-KB9 is the infectious proviral clone of the SHIV-89.6P isolate and was used as the backbone for our SHIV construct.

In this article, we report the construction of SHIV-KBQJ-12, a chimera that expresses most of the *env* gene of the HIV-1 CRF08_BC infectious molecular clone QJ001 in the SHIV-KB9 backbone. SHIV-KBQJ-12 can replicate efficiently in human and Chinese rhesus macaque peripheral blood mononuclear cells (PBMCs) and exclusively uses CCR5 as the coreceptor for cell entry. Intravenous inoculation of SHIV-KBQJ-12 stocks into two Chinese rhesus macaques resulted in productive infection, as evidenced by the detection of viral DNA and RNA in blood and various tissues. The SHIV-KBQJ-12/Chinese rhesus macaque model may be useful to study the pathogenic mechanism of infection of viruses bearing the CRF08_BC envelope and to evaluate the homologous immunoprotective efficacy of Chinese AIDS vaccine candidates.

Materials and methods

Phylogenetic tree analysis

The full-length sequence of HIV-1 QJ001 and the *env* portion of SHIV-KBQJ-12 (HXB2: 6103-8249) were subjected to phylogenetic analysis. Phylogenetic analyses were performed using the neighbor-joining and maximum likelihood methods implemented by MEGA 5.0, and intersubject distances were also calculated [27]. The reliability of the topology of the tree was evaluated by bootstrap analyses with 1000 replicates. The corresponding reference sequences for all clades were obtained from the Los Alamos National laboratory HIV database (http://hiv-web.lanl.gov/).

Construction of the full-length SHIV-KB9 molecular clone

To construct the SHIV-KBQJ-12 molecular clone, we cloned the full-length SHIV-KB9 molecular clone into the vector pBR322 (Invitrogen, New York, NY, USA) using the plasmids 5'SHIV-KB9 and 3'SHIV-KB9 [28]. First, pBR322 was amplified with the primers EE_EcoA and EE_Eag_B, and the polymerase chain reaction (PCR) product was digested with EcoR I and Eag I. Then, 5'SHIV-KB9 was amplified with the primers KB_USEco_A and KB_pbs_B, and the resulting PCR product was digested with EcoR I and Nar I. Next, 5' SHIV-KB9 was digested with Nar I and Sph I, and 3' SHIV-KB9 was digested with Sph I and Not I. Finally, the four fragments described above were ligated to produce the full-length SHIV-KB9 clone, SHIV-KB9/pBR322, which is more stable in STBL3 *E. coli* (Invitrogen).

Construction of the SHIV-KBQJ-12 molecular clone

SHIV-KB9/pBR322 and the HIV-1 CRF08_BC infectious molecular clone, QJ001, were used to construct SHIV-KBQJ-12. Recombinant fragments of these two constructs were produced by the overlap extension method using PrimeStar HS DNA polymerase (Takara,

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Table 1 Primers used for the construction of SHIV-KBQJ-12

Primer	Sequence (5′–3′)
tatSph_A[1]	TTG GCA TGC TGT AGA GCA AGA AAT GGA GCC AGT AG
envSU_B	CAG GTA CCC CAT AAT AGA CTG
vpuPac_A	TGG TTA ATT AAA AGA ATT AGG GAA AGA GCA G
envCS_B	GCC CAT AGT GCT TCC TGC TGC TCC
envCS_A	ATA TGA GGG ACA ATT GGA GAA GTG
envECD_B	AAA GGT GAG TAT CCC TGC CTA ACT C
envECD_A	ATT CAT AAT GAT AGT AGG AGG
SIVnef_B[1]	AAG AGT CAC TGT CGC AGA TCT CC

[1]Primers used for sequencing.

Dalian, China). The sequences of the primers used for the construction are shown in Table 1.

For the Sph I-Pst I fragment, SHIV-KB9/pBR322 was amplified with the primers tatSph_A and envSU_B, and proviral QJ001 DNA was amplified with the primers vpuPac_A and envCS_B. These PCR products were used as templates for the second-round PCR with the primers tatSph_A and envCS_B. The second-round PCR product, which is a chimera of KB9/pBR322 and QJ001, was digested with Sph I and Pst I and then inserted into the pGEM5Zf (-) plasmid vector (Promega, Madison, WI, USA) to produce pKBQJ_SPs.

For the Pst I-Xho I fragment, QJ001 proviral DNA was amplified with the primers envCS_A and envECD_B, and SHIV-KB9/pBR322 was amplified with the primers envECD_A and SIVnef_B. These products were used as templates for the second-round PCR with the primers envCS_A and SIVnef_B. The chimeric product was digested with Pst I and Xho I and inserted into the pBluescriptSK (-) plasmid (Stratagene, La Jolla, CA, USA) to produce pKBQJ_PsX.

The chimeric constructs were analyzed by restriction enzyme digestion and were further confirmed by DNA sequencing. To construct the full-length SHIV-KBQJ-12 proviral clone, pKBQJ_SPs was digested with Sph I and Pst I, and pKBQJ_PsX was digested with Pst I and Xho I; the Sph I-Xho I region of SHIV-KB9/pBR322 was then replaced by the fragments described above.

In addition, the *env* gene fragment (HXB2: 6103-8249) of SHIV-KBQJ-12 was sequenced with the primers tatSph_A and SIVnef_B and subjected to phylogenetic analysis as described above.

Electron microscopy

Electron microscopy analysis was performed by the Center for Analysis and Testing, Wuhan Institute of Virology, Chinese Academy of Sciences, using standard procedures.

Virus assay

The SHIV-KBQJ-12 and SHIV-KB9 plasmids were transfected into 293T cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The virus-containing supernatants were collected after incubating the cells for 2 days at 37°C and quantified by p27 antigen enzyme-linked immunosorbent assay (ELISA) (Advanced Bioscience Laboratories, Rockville, MD, USA). The supernatants were then passed through a 0.45-μm-pore-size filter (Millipore, Boston, MA, USA) to produce cell-free virus stocks and stored in one-ml aliquots at −80°C until use.

The tissue culture infectious dose ($TCID_{50}$) was determined in TZM-bl cells, using standard methods and calculated with the Reed–Muench equation as previously described [29].

Coreceptor utilization assays

Ghost-CD4-CXCR4 and Ghost-CD4-CCR5 cell lines were used to study virus tropism as previously described [21]. Cells express GFP after infection with SHIV strains that use the CXCR4 or CCR5 coreceptor. The cells were plated at a density of 2×10^5 cells per well in 24-well plates and grown in RPMI 1640 with 10% bovine calf serum 24 hours prior to infection. Then, cells were infected overnight with 50 ng of p27*gag* of each virus stock per milliliter. The medium was replaced the following morning. After a 60-hour incubation at 37°C, the cells were washed, trypsinized, and resuspended in 200 μl phosphate-buffered saline (PBS) containing 2% serum, and 20,000 cells were collected and evaluated by flow cytometry. Each experiment was performed in triplicate.

Infectious activity of SHIV clones in human and macaque lymphocyte cells

PBMCs were isolated from whole blood by Ficoll-Paque gradient centrifugation and cultured in the presence of five μg phytohemagglutinin (PHA) per milliliter for 3 days to activate T cells. Next, human or macaque PBMCs were infected overnight with viral supernatant harvested from transiently transfected 293T cells or infected macaque PBMCs; SHIV-KB9 virus was used as a positive control. At zero, three, six, nine, 12, 15, 18, and 21 days post-infection, supernatants were collected for p27*gag* titration using a SIV

p27 antigen ELISA kit. Each experiment was performed in triplicate.

Inoculation and clinical monitoring of animals

Two rhesus monkeys of Chinese origin were used in this study. Both of the animals were seronegative for SIV, simian retrovirus type D (SRV/D), simian T-cell leukemia virus (STLV), and herpes B virus by antibody ELISA and PCR before any experimental procedures. Animal experiments were approved by the Animal Care and Use Committee of the Institute of Laboratory Animal Science, Chinese Academy of Medical Science (ILAS, CAMS), where the experimental animals were housed. Each monkey was inoculated intravenously with 2000 $TCID_{50}$ of SHIV-KBQJ-12 virus stock. The animals were physically examined and monitored regularly for opportunistic infections and other clinical signs of disease over a 14-week period. EDTA-anticoagulated blood samples were drawn from infected animals twice each week in the first month and once each week for the remainder of the study to detect the presence of the virus and analyze the variety of T-cell subsets. On day 98 post-inoculation, the two rhesus monkeys were euthanized, and tissue samples, including groin lymph node, mesenteric lymph node, heart, liver, spleen, lung, kidney, thymus, marrow, cerebrum, spinal cord, ovary, testis, tonsil, oral cavity, fundus, duodenum, jejunum, ileum, cecum, and rectum, were collected and used to confirm the occurrence of the viral infection further and analyze the virus localization.

PCR analysis

Nested PCR was performed on genomic DNA and cDNA from whole blood and various tissues with SIV gag-specific primer pairs as previously described [30]. Genomic DNA was isolated with the DNeasy Blood&Tissue Kit (Qiagen, Hilden, Germany). RNA was extracted from various tissues with the RNeasy Mini kit (Qiagen), and cDNA was then synthesized by one-step reverse transcriptase PCR (RT-PCR) with avian myeloblastosis virus (AMV) reverse transcriptase (Takara).

CD4 cell number analysis

Blood lymphocytes were used in the flow cytometric analysis to determine T-cell subsets using a whole-blood lysis technique. The following monoclonal antibodies (mAbs) from BD Biosciences (Pharmingen, San Diego, CA, USA) were used in this assay: CD3-FITC (cat.556611), CD4-PerCP (cat.550631), and CD8-PE (cat.557086). The results were examined on a FACScan analyzer (Becton-Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Absolute lymphocyte counts in the blood were measured using an automated blood cell counter (F-820; Sysmex, Kobe, Japan).

Results

Phylogenetic tree analysis

To confirm the clade of HIV-1 infectious molecular clone QJ001, we performed a phylogenetic analysis of the full-length genome sequence with various well-known genetic subtypes of HIV. QJ001 was positioned in the cluster of subtype CRF08_BC and was distinct from subtype C and CRF07_BC (Fig. 1A), supporting the construction of SHIV-KBQJ-12.

Construction of SHIV-KBQJ-12 and sequence analysis

The full-length genome of SHIV-KB9 was inserted into the cloning vector pBR322 to generate SHIV-KB9/pBR322. This plasmid, which was used as the backbone for construction of SHIV-KBQJ-12, is more stable in STBL3 *E. coli* because it is a moderate-copy-number vector.

The SHIV-KBQJ-12 clone was constructed by overlap extension PCR and standard cloning techniques. The primary *env* gene, including gp120 as well as the entire extracellular domain, the transmembrane region, and part of the cytoplasmic tail of gp41, was PCR amplified from the plasmid QJ001, which is an HIV-1 CRF08_BC infectious molecular clone, and used to replace the corresponding region of SHIV-KB9 *env* (Fig. 2). SHIV-KBQJ-12 was analyzed by restriction enzyme digestion and further confirmed by DNA sequencing of the *env* gene fragment (HXB2: 6103-8249).

Phylogenetic analysis of the above *env* gene fragment revealed that the *env* gene carried by SHIV-KBQJ-12 belongs to CRF08_BC, a main prevalent and unique subtype of HIV-1 in China, differentiating it from subtype C and CRF07_BC (Fig. 1B).

The SHIV-KBQJ-12 virus stock was produced by transfecting 293T cells with the verified plasmid for the subsequent experiments. The viral supernatant was collected, and the level of the p27*gag* antigen was measured. Cells were harvested, and the intact virus particles in the 293T cells were observed by electron microscopy (Fig. 3).

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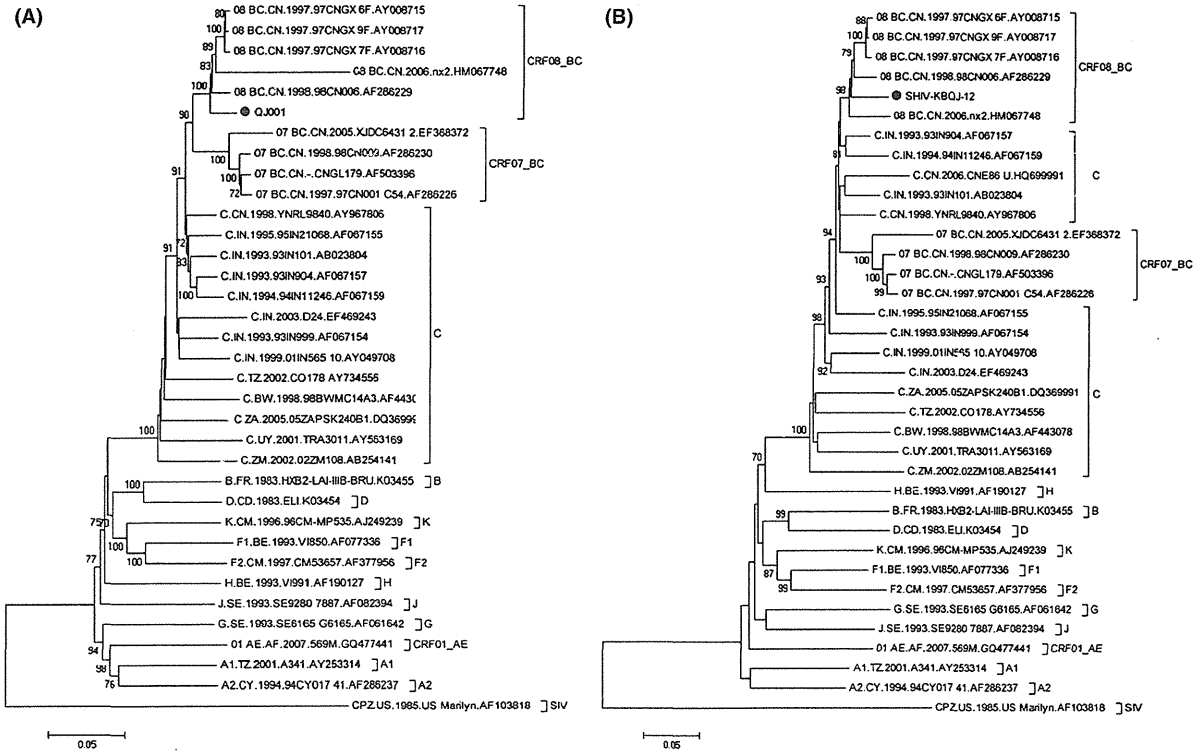

Fig. 1 Phylogenetic analysis of the full-length sequence of HIV-1 QJ001 (A) and the *env* portion of SHIV-KBQJ-12 (HXB2: 6103-8249) (B). Both of the above two sequences were used to build a neighbor-joining phylogenetic tree with the corresponding sequences of the reference virus isolates, which were obtained from the Los Alamos National laboratory HIV database (http://hiv-web.lanl.gov/). Significant bootstrap values (>70%) are indicated next to the nodes.

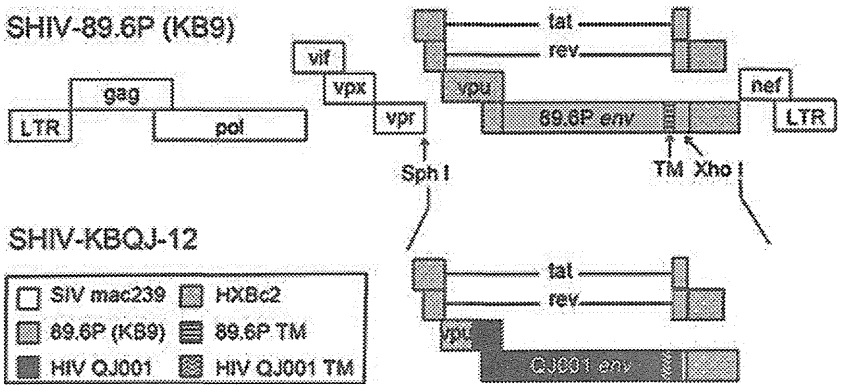

Fig. 2 Schematic representation of the construction of SHIV-KBQJ-12. Genetic construction of SHIV-KBQJ-12 was performed by replacing the 2.6-kb SphI-XhoI fragment of SHIV-KB9 with the new chimeric SphI-XhoI fragment, which was produced by the overlap extension method using SHIVKB9/pBR322 and HIV-1 QJ001 as templates. The resulting sequence derived from HIV-1 QJ001 (HXB2: 6103-8249) spans gp120 as well as the entire extracellular domain, the transmembrane region (TM), and part of the cytoplasmic domain of gp41.

Coreceptor usage of SHIV-KBQJ-12

We determined the coreceptor usage of SHIV-KBQJ-12 using GHOST cells expressing CXCR4 or CCR5; SHIV-KB9 was used as a control (Table 2). SHIV-KB9 has been reported to use CCR5 and CXCR4 as its coreceptor for entry, and we confirmed that SHIV-KB9 replicated not only in CCR5 cells but also in CXCR4 cells. However, productive infection of SHIV-KBQJ-12 occurred only in CCR5 cells, indicating that

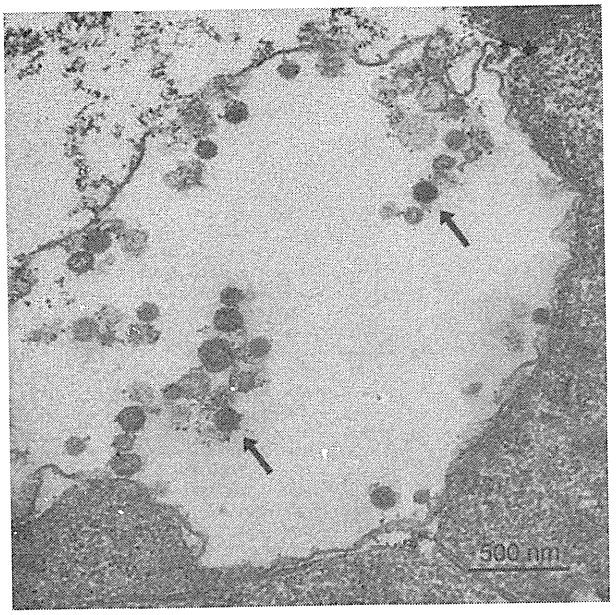

Fig. 3 Electron microscopy examination of SHIV-KBQJ-12-transfected 293T cells. The arrow indicates the virus particle.

Table 2 Coreceptor usage of SHIV-KBQJ-12

	GHOST cell	
Virus	CXCR4	CCR5
SHIV-KB9	+	+
SHIV-KBQJ-12	–	+
Mock-infected	–	–

GHOST cell lines expressing CXCR4 or CCR5 were infected overnight with cell-free virus stocks. At 60 hours post-infection, cells were collected and analyzed by flow cytometry to determine the virus tropism. The data shown are representative of three independent experiments. –, no infection (<1%); +, robust infection (>1%).

SHIV-KBQJ-12 exclusively uses CCR5 as a coreceptor for entry.

Replication of SHIV-KBQJ-12 in human and Chinese rhesus macaque PBMCs

To evaluate the growth of SHIV-KBQJ-12 in human PBMCs, we used the virus stock to infect previously PHA-stimulated human PBMCs; SHIV-KB9 was used as the positive control (Fig. 4A). Culture supernatants were collected every 3 days after infection and quantified by p27 antigen ELISA for up to 30 days. Both of the viruses were able to replicate efficiently in human PBMCs, with peak p27 production in the supernatant observed at day nine post-infection. Moreover, the replication level of SHIV-KBQJ-12 was extremely similar to that of SHIV-KB9.

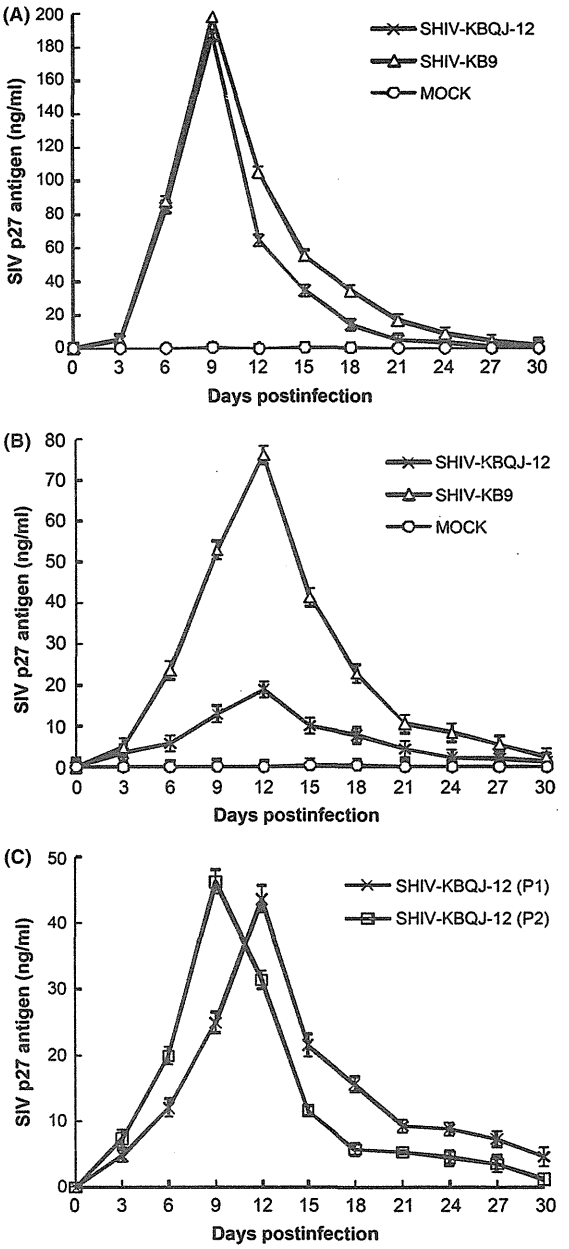

Fig. 4 Replication kinetics of SHIV-KBQJ-12 in human and rhesus macaque PBMCs. Human (A) and rhesus macaque (B) PBMCs were infected with a SHIV-KBQJ-12 viral stock generated from transfection of 293T cells with SHIV-KBQJ-12 proviral DNA using SHIV-KB9 as the positive control. (C) Serial passage of SHIV-KBQJ-12 in rhesus macaque PBMCs. The supernatant of SHIV-KBQJ-12 at day 12 post-infection was collected and used as SHIV-KBQJ-12 (P1). Similarly, the supernatant of SHIV-KBQJ-12 (P1) at day 12 post-infection was collected and used as SHIV-KBQJ-12 (P2). PBMC supernatants were monitored by quantifying p27 levels over time. The data shown are the mean for two wells, and error bars indicate the values for the two wells. The data shown are representative of three independent experiments.

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Both of the SHIV strains also replicated well in Chinese rhesus macaque PBMCs, and p27 reached a peak concentration in the culture supernatant at day 12 post-infection (Fig. 4B). The peak p27 concentration of SHIV-KBQJ-12 was 18.9 ng/ml. To enhance the adaptability of SHIV-KBQJ-12, we further passaged the virus in macaque PBMC. Accordingly, the supernatant of SHIV-KBQJ-12 at day 12 post-infection was collected and used to infect fresh PHA-stimulated macaque PBMCs, resulting in a peak p27 concentration of SHIV-KBQJ-12 (P1) of 43.6 ng/ml at day 12 post-infection (Fig. 4C). The same protocol was performed with SHIV-KBQJ-12 (P2), yielding a viral peak of 46.2 ng/ml at day nine post-infection; thus, the peak concentration was reached much higher and faster with P2 than P1.

Based on these data, we concluded that SHIV-KBQJ-12 can productively infect human and Chinese rhesus macaque PBMCs and that the adaptability of SHIV-KBQJ-12 is enhanced by two passages in macaque PBMCs.

Infectious activity in Chinese rhesus macaques

Rhesus macaques are the most commonly used non-human primates for HIV research. Because we had established that Chinese rhesus macaque PBMCs could be productively infected *in vitro*, we next attempted to inoculate Chinese rhesus macaques with SHIV-KBQJ-12. A male juvenile rhesus macaque, designated 091270, and a female juvenile rhesus macaque, designated 090749, were inoculated intravenously with SHIV-KBQJ-12 stocks and monitored for infection. Nested PCR was performed on genomic DNA isolated from whole blood at different time points using SIV *gag*-specific primer pairs. The first positive result was observed on day 12 post-inoculation in macaque 091270 and on day six post-inoculation in macaque 090749. In addition, the nested PCR continued to yield positive results until the day of sacrifice. The plasma viral RNA loads of the two rhesus macaques were also determined by quantitative reverse transcriptase PCR (qPCR), yielding negative results (data not shown). Therefore, both of the exposed macaques were systemically infected. In the two animals, absolute peripheral $CD4^+$ T-cell numbers remained within the normal range during the 14-week infection period (Fig. 5A). There were also no obvious changes observed in CD4/CD8 ratios over this period (Fig. 5B). The animals maintained their weight and appetite and had no signs of clinical AIDS (data not shown).

To assess the replication of SHIV-KBQJ-12 in tissues, the two inoculated rhesus macaques were sacrificed at day 98 post-infection. The corresponding PCR was performed on DNA or RNA extracted from various tissues, and the result revealed that positive *gag* PCR amplification of DNA appeared in the cerebrum, groin lymph node, ileum, jejunum, marrow, mesenteric lymph node, rectum, and spleen of both macaques, with additional positive results in the fundus, heart, and testis of macaque 091270 and in the duodenum, ovary, thymus, and tonsil of macaque 090749. According to the RNA results, the virus existed in the cerebrum, groin lymph node, marrow, mesenteric lymph node, rectum, and spleen of both macaques. In addition, the fundus, heart, and testis of macaque 091270 and the ileum, ovary, and tonsil of macaque 090749 were shown to be infected (Table 3).

Based on these results, we summarize that SHIV-KBQJ-12 can productively infect two Chinese rhesus macaque intravenously and is distributed mainly in the genital system, central nervous system, and partial gut-associated lymphoid tissues.

Discussion

In this study, we constructed an R5-tropic SHIV-KBQJ-12, a chimera that bears a majority of envelope glycoprotein from the HIV-1 CRF08_BC infectious

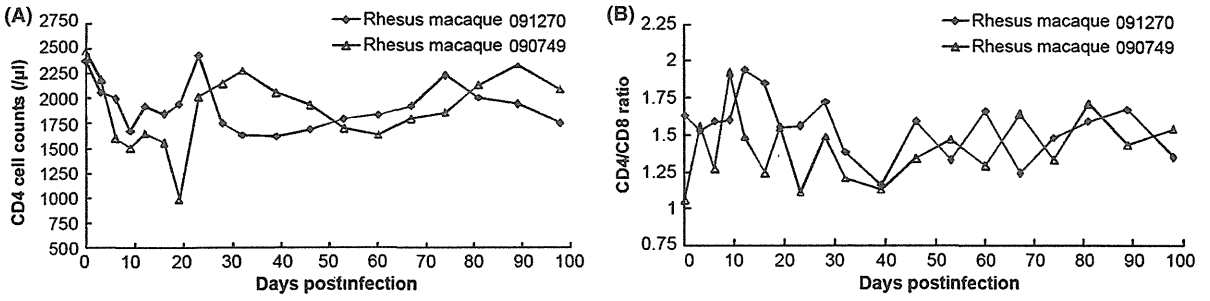

Fig. 5 Productive infection of SHIV-KBQJ-12 *in vivo*. Two Chinese rhesus macaques, 091270 and 090749, were each intravenously inoculated with 2000 $TCID_{50}$ of cell-free virus stock. Peripheral blood at different time points was collected and analyzed for changes in absolute $CD4^+$ T-cell counts (A) and CD4/CD8 counts (B).

Table 3 Virus distribution in various tissues

	Rhesus Macaque 091270		Rhesus Macaque 090749	
Samples	DNA	RNA	DNA	RNA
Cerebrum	+	+	+	+
Groin lymph node	+	+	+	+
Marrow	+	+	+	+
Mesenteric lymph node	+	+	+	+
Rectum	+	+	+	+
Spleen	+	+	+	+
Ileum	+	–	+	+
Fundus	+	+	–	–
Heart	+	+	–	–
Testis	+	+	ND	ND
Jejunum	+	–	+	–
Ovary	ND	ND	+	+
Tonsil	–	–	+	+
Duodenum	–	–	+	–
Thymus	–	–	+	–
Cecum	–	–	–	–
Kidney	–	–	–	–
Liver	–	–	–	–
Lung	–	–	–	–
Oral cavity	–	–	–	–
Spinal cord	–	–	–	–

ND, not done.

Two rhesus macaques intravenously inoculated with SHIV-KBQJ-12 were sacrificed at day 98 post-infection. The PCR amplification of SIV *gag* was performed using DNA and RNA extracted from various tissue samples to analyze the virus localization. + refers to a positive *gag* PCR amplification result; –refers to a negative *gag* PCR amplification result.

molecular clone QJ001, which comprises the determinant regions for viral entry and phenotype. Moreover, the novel SHIV-KBQJ-12 could establish productive infection not only in human and Chinese rhesus macaque PBMCs *in vitro* but also in two Chinese rhesus macaques *in vivo*.

Previous studies have shown that most *env* genes amplified from clinical specimens do not possess biological activity, and extensive screening must be conducted to obtain the infectious *env* gene for SHIV construction. The *env* gene of SHIV-KBQJ-12 is derived from the HIV-1 CRF08_BC infectious molecular clone QJ001, whose biological function has been established, increasing the speed and probability of obtaining a new SHIV infectious molecular clone. This is also the first study to acquire the *env* gene from an HIV infectious molecular clone and not from a clinical HIV strain to construct SHIV. The use of this gene may also explain why the replication level of SHIV-KBQJ-12 is extremely similar to that of SHIV-KB9 in human PBMCs. Moreover, phylogenetic analysis of the *env* gene further confirmed that SHIV-KBQJ-12 was CRF08_BC, one of the most prevalent subtypes in the Chinese pandemic, and was not identical to subtype C and CRF07_BC, although highly similar to clade C and CRF07_BC. Consequently, SHIV-KBQJ-12 may be more suitable to evaluate candidate vaccines targeting HIV-1 CRF08_BC Env than the currently available HIV-1 subtype C or CRF07_BC envelope-based SHIV strains.

Moreover, the plasmid containing the full-length genome of SHIV is unstable, and it is difficult to prepare substantial virus stocks due to the long terminal repeat (LTR) sequences. The plasmid SHIV-KB9 provided by the HIV/AIDS Reagent Program consists of two plasmids that contain gene fragments from the 5′LTR to the vpr gene and from the tat gene to the 3′LTR, respectively [28]. Here, we constructed the plasmid SHIV-KB9/pBR322, which is stable in STBL3 *E. coli* and can be used to produce the virus stock easily in large quantities. The use of this plasmid will facilitate the construction of new SHIV plasmids with different HIV-1 strains-derived *env* genes and the analysis of these new SHIV viruses.

Unfortunately, the plasma viral RNA loads in the two rhesus macaques could not be detected by qPCR after inoculation with SHIV-KBQJ-12. The fundamental reason is the replication ability of SHIV-KBQJ-12, which has not been passaged continuously in rhesus macaques, may be less effective to produce massive viruses in macaques. Therefore, the number of plasma viral RNA copies is lower than the lower limit of qPCR, which is 50 viral RNA copies/ml of plasma. Importantly, productive infection of two rhesus macaques was confirmed by corresponding PCR amplification in whole blood and various tissue samples. These results also indicate that SHIV may be distributed in some tissues even though the plasma viral load is low. In addition, SHIV was clearly distributed in the genital system, central nervous system, and partial gut-associated lymphoid tissues, which may serve as reservoirs of viruses. This may deepen our knowledge of the host–virus interaction and the pathogenicity of HIV-1.

Neither of the two rhesus macaques experienced a dramatic decline in the $CD4^+$ lymphocyte count during the 14-week observation period. This result is not totally unexpected because the SHIVs that have been associated with significant $CD4^+$ lymphopenia and with disease are either dual tropic or use CXCR4 as the coreceptor for cell entry. In contrast to the dual tropism of SHIV-KB9, which can induce acute, severe pathogenicity and has been widely used as a challenge virus in the SHIV/rhesus macaque model, SHIV-KBQJ-12 is one of only a few CCR5-utilizing SHIVs

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that have been constructed. The biological characteristics of SHIV-KBQJ-12 may allow it to mimic HIV disease progression in humans without eliciting acute pathogenicity. Thus, it will be interesting to ascertain whether the infected macaques develop $CD4^+$ T-lymphocyte destruction and clinical AIDS-like disease on long-term follow-up. However, long-term adaptation of SHIV viruses in juvenile rhesus macaques and rapid passage has been shown to induce the emergence of pathogenic variants. The serial passages of SHIV-KBQJ-12 in rhesus macaque PBMCs resulted in more effective and rapid replication capacity; hence, it is worthwhile to enhance the virulence and pathogenicity of SHIV-KBQJ-12 through macaque continuous passage.

In summary, because no pathogenic SHIV virus strain carrying the HIV-1 B'/C subtype *env* gene is available, it is impossible to evaluate the ability of Chinese anti-HIV-1 vaccine candidates to provide homologous protection from disease. In the present study, we generated SHIV-KBQJ-12, a CRF08_BC-based SHIV chimera that utilizes the CCR5 chemokine receptor as the coreceptor for cell entry. SHIV-KBQJ-12 chimeric viruses efficiently replicate in human and rhesus macaque PBMCs. We also established a Chinese rhesus macaque infection model using SHIV-KBQJ-12. Although the SHIV-KBQJ-12/macaque model described here is non-pathogenic, studies of other successful SHIV virus strains suggest that it may gain virulence and pathogenicity through continuous passage in rhesus macaques. In fact, we are now attempting to continuously passage the virus SHIV-KBQJ-12 in macaques until a novel and pathogenic CRF08_BC SHIV is obtained, although this may be a lengthy process. We hope that this chimera will become a useful tool to study viral pathogenesis and to test the efficacy of vaccine candidates targeting HIV-1 CRF08_BC *env*.

Sequence data

The nucleotide sequence of HIV-1 QJ001 has the GenBank accession number KC914396.

Acknowledgments

We thank the Key Laboratory on Emerging Infectious Diseases and Biosafety in Wuhan. This work was supported by a grant of the Key Projects in the National Science & Technology Pillar Program during the twelfth Five-Year Plan Period of China (2012ZX10001006) and 135 Program of Wuhan Institute of Virology, Chinese Academy of Sciences (Y206513SA2).

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GASTROENTEROLOGY 2013;145:447–455

BASIC AND TRANSLATIONAL—LIVER

Neutralizing Antibodies Induced by Cell Culture–Derived Hepatitis C Virus Protect Against Infection in Mice

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See editorial on page 285.

BACKGROUND & AIMS: Hepatitis C virus (HCV) infection is a major cause of liver cancer, so strategies to prevent infection are needed. A system for cell culture of infectious HCV particles (HCVcc) has recently been established; the inactivated HCVcc particles might be used as antigens in vaccine development. We aimed to confirm the potential of HCVcc as an HCV particle vaccine. **METHODS:** HCVcc derived from the J6/JFH-1 chimeric genome was purified from cultured cells by ultrafiltration and ultracentrifugation purification steps. Purified HCV particles were inactivated and injected into female BALB/c mice with adjuvant. Sera from immunized mice were collected and their ability to neutralize HCV was examined in naive Huh7.5.1 cells and urokinase-type plasminogen activator–severe combined immunodeficiency mice ($uPA^{+/+}$-SCID mice) given transplants of human hepatocytes (humanized livers). **RESULTS:** Antibodies against HCV envelope proteins were detected in the sera of immunized mice; these sera inhibited infection of cultured cells with HCV genotypes 1a, 1b, and 2a. Immunoglobulin G purified from the sera of HCV-particle-immunized mice (iHCV-IgG) inhibited HCV infection of cultured cells. Injection of IgG from the immunized mice into $uPA^{+/+}$-SCID mice with humanized livers prevented infection with the minimum infectious dose of HCV. **CONCLUSIONS: Inactivated HCV particles derived from cultured cells protect chimeric liver $uPA^{+/+}$-SCID mice against HCV infection, and might be used in the development of a prophylactic vaccine.**

Keywords: HCV Particle; HCV Vaccine; Immunization; Virology.

Hepatitis C virus (HCV), an enveloped virus that belongs to the *Hepacivirus* genus of the *Flaviviridae* family, is a human pathogen that is a major cause of chronic hepatitis, cirrhosis, and hepatic carcinoma. HCV therapy mainly involves treatment with pegylated interferon and ribavirin. However, these agents are not very effective for patients with high titer HCV-RNA and genotype 1. Thus, it is necessary to develop new, more effective therapies and preventive care treatments for HCV infection.

A cell culture-derived HCV (HCVcc) was developed using the genome of the genotype 2a JFH-1 strain. HCVcc was shown to be infectious in vitro and in vivo,[1–3] and its analysis has made it possible to understand the HCV lifecycle. Thus, HCVcc can be considered as a useful model for the screening of new anti-HCV drugs. However, the fact that only human beings and chimpanzees are susceptible to HCV has greatly slowed the in vivo study. As a tool for circumventing this problem, a human liver chimeric albumin enhancer/promoter urokinase plasminogen activator-severe combined immunodeficiency (uPA-SCID) mouse was established.[4] Thus, the HCV cell culture system and the human liver chimeric mouse are potent tools for pharmaceutical research concerning HCV.

Prevention of HCV infection is important for control of the pathogenesis of hepatitis C. However, there is no commercial vaccine for HCV at present, although vaccines are now being developed using HCV components, peptides, DNA, and viral vectors.[5] Because HCVcc is a structural mimic of the native HCV particle, it has the potential to function as an immunogen for the development of an anti-HCV vaccine. In this study, to use HCVcc as an HCV-particle vaccine, we purified HCVcc and determined the neutralizing effects of the HCV-particle vaccine against HCV infection in mice.

Materials and Methods

Cell Culture

Huh7 and Huh7.5.1 cells[2] (a generous gift from Dr Francis V. Chisari) were cultured in 5% CO_2 at 37°C in Dulbecco's modified

Abbreviations used in this paper: Cont, control; DMEM, Dulbecco's modified Eagle medium; Frac, fraction; HCV, hepatitis C virus; HCVcc, cell-cultured hepatitis C virus; HCVpp, hepatitis C virus pseudoparticle; IC50, 50% inhibitory concentration; iHCV, vaccine immunized hepatitis C virus; rE1, recombinant E1; rE2, recombinant E2; RTD-PCR, real-time detection reverse-transcription polymerase chain reaction; SCID, severe combined immunodeficiency; uPA, albumin enhancer/promoter urokinase plasminogen activator.

0016-5085/$36.00
http://dx.doi.org/10.1053/j.gastro.2013.05.007

Eagle medium (DMEM) containing 10% fetal bovine serum (DMEM-10) as previously reported.[1]

Plasmids

pJ6/JFH1 was generated by replacing the JFH-1 structural region with that of the J6CF strain as previously reported.[6]

HCV Particle Purification

J6/JFH-1 chimeric HCVcc was purified by ultrafiltration and ultracentrifugation. The methods for HCVcc production and purification were as described in the Supplementary Materials and Methods section.

Infectivity Titration

Huh7.5.1 cells were used to determine the viral infectivity titer using end-point dilution and immunofluorescence methods as described previously.[7] The titer was expressed as focus-forming units per milliliter.

HCV Pseudo-Particle Production

Murine leukemia virus pseudotypes were generated according to methods described previously.[8] HCV pseudo-particle (HCVpp) harboring the envelope of the genotype 1a (H77[9]), 1b (TH[10]), or 2a (J6CF[11]) strain were generated as described in the Supplementary Materials and Methods section.

Preparation of Recombinant Proteins

HCV-E1 and HCV-E2 recombinant proteins were generated as described in the Supplementary Materials and Methods section.

Immunization of Mice With the HCV Vaccine

Mice were immunized as described in the Supplementary Materials and Methods section.

Enzyme Immunoassay

Anti-E1 and anti-E2 antibodies were detected by enzyme immunoassay for stabilized recombinant J6E1/FLAG and J6E2/FLAG protein, respectively, as described in the Supplementary Materials and Methods section.

HCV Inhibition Assay

Inhibition of HCV infection in cultured cells was assayed using HCVpp and HCVcc for infection as described in the Supplementary Materials and Methods section.

IgG Purification

Mouse IgG was purified using KAPTIV-GY resin (Technogen, Tehran, Iran) as described in the Supplementary Materials and Methods section.

Anti-HCV Envelope IgG Adsorption

Anti-HCV envelope antibodies were absorbed using recombinant FLAG-tagged HCV-E1 and HCV-E2 proteins. Briefly, 1 mg/mL IgG from HCV-particle-immunized mice (iHCV-IgG) was precleared with anti-FLAG M2 agarose, and then mixed with 50 μg each of J6E1/FLAG and J6E2/FLAG protein. The IgG recombinant protein mixture was incubated at 4°C overnight, and anti-FLAG M2 agarose then was added into the mixture, which was centrifuged at 3000 × *g* for 2 minutes and the supernatant was collected as anti-envelope IgG-adsorbed IgG.

Inhibition of HCV Infection in Chimeric Mice in Which the Liver Was Repopulated With Human Hepatocytes

SCID mice that were transgenic for the urokinase-type plasminogen activator gene and in which the liver was repopulated with human hepatocytes (chimeric mice) were purchased from Phoenix Bio Co, Ltd (Hiroshima, Japan). Only mice showing levels of human albumin greater than 7 mg/mL were used in the experiments. The mice were injected intraperitoneally with 100 μg of control (Cont)-IgG or iHCV-IgG at 1 week and at day -1 before virus challenge. J6/JFH-1 HCVcc particles (10^3, 10^4, and 10^5 RNA copies/mouse) were mixed with 50 μg/mL of Cont-IgG or iHCV-IgG, and used to challenge the mice. Blood samples were collected within 1 week, and HCV RNA was determined using real-time detection reverse-transcription polymerase chain reaction (RTD-PCR).

Western Blot Analysis

Purified HCV particle samples were resolved on 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to an Immobilon-P membrane (Millipore, Billerica, MA). Western blot was performed using an anti-E1 or an anti-E2 antibody as described in the Supplementary Materials and Methods section.

Statistical Analysis

The Dunnett test was used to compare the statistical significance of differences between groups of data. A P value less than .05 was considered significant. Data are reported as means ± SEM as indicated.

Results

HCV Particle Purification

HCV particles for mouse immunization were obtained using the cell culture system described in the Materials and Methods section. J6/JFH-1 chimeric HCV was secreted into culture medium supplemented with 2% fetal bovine serum, which was collected and centrifuged; the supernatant was concentrated by ultrafiltration using a 500-kilodalton cut-off membrane. The concentrated culture fluid was diafiltered using phosphate-buffered saline. These purification procedures removed approximately 90% of the contaminating proteins in the solution of virus particles. Sucrose cushion or gradient centrifugation then was performed to purify the virus particles further. After each procedure, the fractionated viruses were concentrated by ultrafiltration and the amount of HCV core and RNA, the concentration of total protein, and viral infectivity were determined (Supplementary Table 1). As shown in Figure 1, HCV particles, which had 2 distinct characteristics, were obtained in different fractions after sucrose gradient fractionation. One fraction (Fraction [Frac] 6) had low infectivity and a relatively high level of HCV core and RNA, and the other fraction (Frac 8) had high infectivity and a lower level of HCV core and RNA. Thus, the yield of HCV particles was highest in sucrose gradient Frac 6 (Supplementary Table 1), whereas Frac 8 displayed

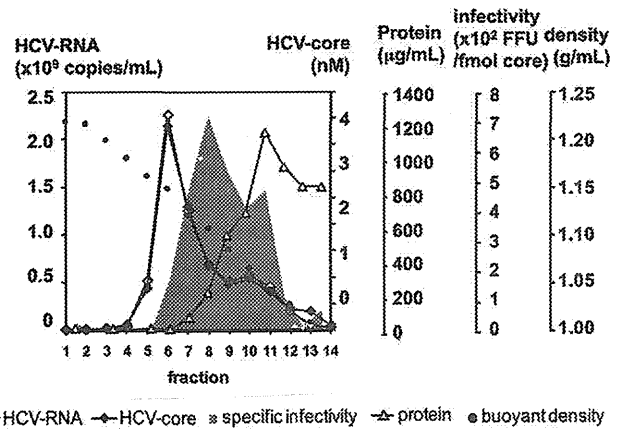

Figure 1. HCV-particle purification from cell cultures. Huh7 cells were inoculated with J6/JFH-1 chimeric HCVcc (multiplicity of infection, 0.2) and infected cells were passaged into CellSTACK (Corning, Corning, NY). The cell supernatant was collected and concentrated by ultrafiltration using a hollow fiber UFP-500-C-3MA (GE Healthcare, Little Chalfont, United Kingdom). Concentrated virus then was diafiltered using phosphate-buffered saline, and the viral fluid was layered on top of a preformed continuous 10%–60% sucrose gradient in 10 mmol/L Tris, 150 mmol/L NaCl, 0.1 mmol/L EDTA. The gradients were centrifuged using an SW28 rotor (Beckman Coulter, Fullerton, CA) at 28,000 rpm for 4 hours at 4°C, and 1-mL fractions were collected from the bottom of the tube. The level of the HCV core protein, HCV RNA, infectivity toward naive Huh7.5.1 cells, total protein, and buoyant density were determined for each fraction. Infectivity is indicated as specific infectivity, which was calculated as the infectivity titer (focus-forming unit [FFU]) divided by the HCV core protein (fmol).

approximately 7-fold higher infectivity than Frac 6 (Table 1). After sucrose cushion purification, the total protein level of the solution of purified virus particles was reduced to less than 0.03% of that of the initial cell culture supernatant (Supplementary Table 1).

Immunization of Mice With HCV Particles

We next immunized BALB/c mice with 2 or 5 pmol of HCV particles that were purified through a sucrose cushion (termed *cushion-2* and *cushion-5*, respectively) or with the HCV particles (2 pmol HCV core) from the earlier-described sucrose gradient fractions 6 or 8, and confirmed the immunogenicity of these particles. Before immunization, the purified HCV particles were inactivated by ultraviolet irradiation and conjugated with monophosphoryl lipid A plus trehalose-6,6-dimycolate. The HCV particles then were injected intraperitoneally into female BALB/c mice and blood samples were collected. Immunization was repeated 4 times at 2-week intervals, and the presence of anti-E1 and anti-E2 antibodies in immunized mouse serum was analyzed using an enzyme immunoassay. Both anti-E1 and anti-E2 antibodies were induced in mice immunized with the HCV particles and the efficiency of antibody induction was the same regardless of the nature of the antigen (Figure 2*A* and *B*).

To confirm that the sera from these immunized mice could inhibit HCV infection, an HCVpp inhibition assay was performed. In this assay, sera from HCV-particle-immunized mice inhibited HCVpp infection of naive Huh7.5.1 cells by HCV strains H77 (1a), TH (1b), and J6CF (2a) (Figure 2*C–E*). Furthermore, the inhibitory effects of different amounts (2 or 5 pmol) of HCV core purified by the sucrose cushion method or of HCV particles from different fractions obtained by sucrose gradient purification were not significantly different. Inhibitory effects of the serum from immunized mice were detected against TH/JFH-1 (genotype 1b) and J6/JFH-1 (genotype 2a) HCVcc infection (Supplementary Figure 1). The 50% inhibitory dilution titer of Frac 6 vaccine-immunized mouse serum was 1/181 and 1/614, and that of Frac 8 vaccine-immunized mouse serum was 1/432 and 1/766 for TH/JFH-1 and J6/JFH-1 HCVcc, respectively. These results indicate that the neutralizing effects of the serum from immunized mice were intergenotypic, and that 2 pmol of the HCV core was a sufficient dose for HCV-particle immunization. In contrast, immunization with saline or bovine serum albumin and adjuvant did not induce any neutralizing effects in mice (Figure 2*C–E*). We used bovine serum albumin as a control because albumin was assumed to be the main contaminating protein in the purified HCV particles (Supplementary Figure 2).

The HCV-Particle Vaccine Induced Anti-HCV Antibody More Efficiently Than an HCV-Component Vaccine

For comparative study of HCV-component and HCV-particle vaccines, recombinant E1 and E2 proteins were produced and used to immunize mice using the same adjuvant as that used earlier for the HCV-particle vaccine. These recombinant FLAG-tagged J6E1 and J6E2 proteins (J6E1/FLAG and J6E2/FLAG) were expressed and secreted into the culture supernatant of COS-1 cells, and were purified by affinity chromatography using M2-agarose. Before injection into mice, the purified recombinant proteins, as well as the inactivated HCV particles obtained from the sucrose gradient fractionation described earlier, were analyzed by Western blot using anti-E1 and anti-E2 antibodies, and the amount of E1 or E2 protein present in the HCV particles was determined. Densitometric analysis of the immunoblot shown in Figure 3 indicated that an aliquot of HCV particles that contained 1 pmol of HCV core protein included approximately 10 ng each of

Table 1. Composition of Purified HCV Particles After Ultracentrifugation

	Core/RNA, fmol/copy	Infectivity/core, FFU/fmol	Infectivity/RNA, FFU/copy	Core/protein, fmol/μg
Frac 6	6.54×10^{-7}	129.8	8.50×10^{-5}	113.8
Frac 8	5.38×10^{-7}	987.9	5.32×10^{-4}	19.3
Ratio (Frac 8/Frac 6)	0.82	7.61	6.26	0.17

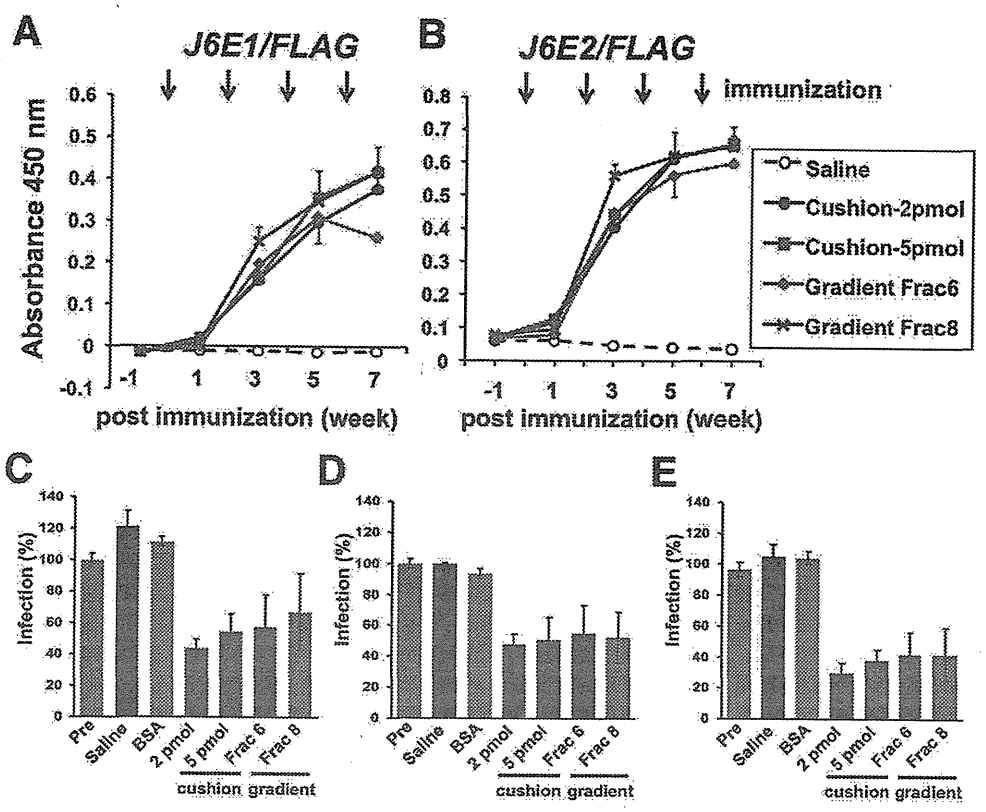

Figure 2. Cross-genotypic neutralizing effect of the sera from HCV-particle–immunized mice. HCV particles that were purified using a sucrose cushion (2 or 5 pmol HCV core) or sucrose gradient fractionation (Frac 6 and Frac 8, 2 pmol HCV core each) were inactivated by ultraviolet irradiation. The inactivated HCV -particles were conjugated with Sigma Adjuvant System (Sigma, St Louis, MO), and were injected intraperitoneally into BALB/c mice (5 weeks old, female, n = 3). Saline or bovine serum albumin (150 μg) with adjuvant was injected into mice as a control. Immunization was repeated 4 times at 2-week intervals (at weeks 0, 2, 4, and 6). Blood was collected at weeks 1, 3, 5, and 7 after immunization. Pre-immune sera and immunized mouse sera were prepared from these blood samples and were heat-inactivated at 56°C for 1 hour. The heat-inactivated sera were diluted (1000-fold) and their reactivity with (*A*) J6E1/FLAG and (*B*) J6E2/FLAG recombinant proteins was analyzed using an enzyme immunoassay. Assays were performed in triplicate. The *y*-axis indicates absorbance at 450 nm. Mean values ± SEM are shown. Mouse serum-mediated inhibition of HCV infection was assayed using HCVpp genotypes (*C*) 1a (H77), (*D*) 1b (TH), and (*E*) 2a (J6CF). The sera were diluted (100-fold) and HCVpp infection in naive Huh7.5.1 cells was examined. Assays were performed in triplicate and infection rates are expressed as mean ± SEM.

the E1 and E2 proteins. Thus, HCV particles that contained 2 pmol of HCV core contained approximately 20 ng of each envelope protein.

Recombinant J6E2/FLAG was inoculated into mice at a concentration of 20, 200, or 2000 ng/mouse or was inoculated together with the same dose of J6E1/FLAG (20 or 200 ng/mouse). Inactivated HCV particles from Frac 6 or Frac 8 were injected into mice using 2 pmol (20 ng each of E1 or E2) of HCV core protein per mouse. Sera from immunized mice were collected and the presence of anti-E2 antibodies was analyzed using an enzyme immunoassay. Anti-E2 antibodies were induced in all immunized mice, and the highest level of anti-E2 antibodies was induced in the group of mice that were immunized with 200 or 2000 ng of the J6E2 protein per mouse. On the other hand, the level of anti-E2 antibodies that was induced by immunization with 20 ng of the inactivated HCV particles was higher than that induced by the same amount of recombinant envelope proteins (Figure 4*A*). To analyze the inhibitory effects of the sera from immunized mice against HCV infection, HCVpp was incubated with heat-inactivated mouse sera, and then was used to inoculate Huh7.5.1 cells. Sera from HCV-particle–immunized mice were able to inhibit HCVpp infection; however, sera from mice immunized with 20 or 200 ng of the recombinant E2 or E2 plus E1 proteins did not show significant neutralization activity. Although sera from mice immunized with 2 mg of the recombinant E2 protein could neutralize HCVpp infection, the sera from mice immunized with only 20 ng of the HCV particles from sucrose gradient Frac 6 or Frac 8 also were able to induce similar levels of anti-envelope antibody and HCV neutralization (Figure 4*B*). These results suggest that HCV particles have a greater potential as vaccine candidates than recombinant envelope proteins in terms of their potency for antibody induction.

Vaccination With Cell-Cultured HCV Induces Anti-HCV IgG

As described earlier, inactivated HCV particles can induce an anti-HCV effect in the sera of immunized mice. To confirm that this anti-HCV effect was dependent on HCV-specific antibodies, IgG was purified from the sera of Saline- or HCV-particle-immunize mice, respectively, and was assayed in an HCV inhibition assay. The purity of the

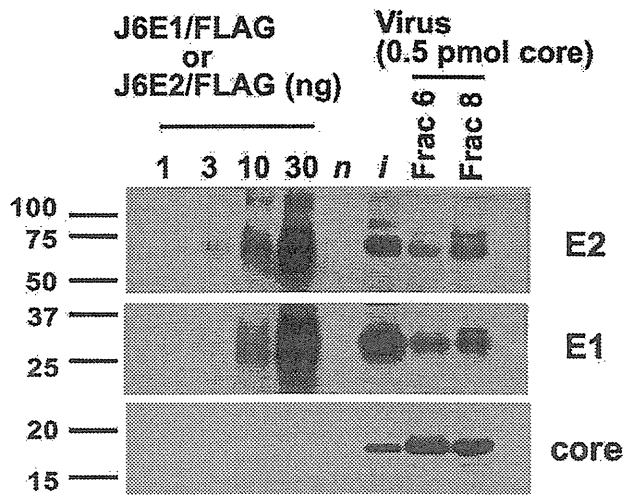

Figure 3. Western blot analysis of envelope proteins in purified HCV particles. The HCV core (0.5 pmol) of purified HCV particles (Frac 6 and Frac 8), as well as 1–30 ng of recombinant envelope proteins (J6E1/FLAG or J6E2/FLAG) were resolved on 12% sodium dodecyl sulfate–polyacrylamide gels and transferred to a polyvinylidene difluoride membrane. Naive Huh7 cells and J6/JFH-1–infected Huh7 cells were lysed using Passive Lysis Buffer (Promega, Madison, WI), and were assayed similarly as negative (*n*) and positive (*i*) controls, respectively. The membrane was blocked and probed with primary antibodies against the virus core (2H9; 3 μg/mL), E1 (B7567; 10 μg/mL), and E2 (AP33; 3 μg/mL) proteins. The membrane then was probed with horseradish-peroxidase–conjugated secondary antibody, and bound antibody was detected using ECL-plus (GE Healthcare, Little Chalfont, UK).

purified IgG was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis (Figure 5*A*). Purified IgGs were pre-incubated with J6/JFH-1 HCVcc and this mixture subsequently was used to inoculate Huh7.5.1 cells. The IgG from HCV-particle-immunized mice (iHCV-IgG) inhibited HCV infection in an iHCV-IgG dose-dependent manner, whereas the IgG from saline-immunized mice (Cont-IgG) did not show any neutralizing effects on this in vitro assay (Figure 5*B*). The 50% inhibitory concentration (IC50) of iHCV-IgG against HCVcc infection was 16.1 μg/mL. The iHCV-IgG from Frac 6– and Frac 8–immunized mice showed a neutralizing effect on TH (genotype 1b) and J6CF (genotype 2a) HCVpp infections (Supplementary Figure 3); the IC50 values were 18.0 (TH) and 30.4 (J6CF) μg/mL for Frac 6–IgG, and 15.1 (TH) and 33.1 (J6CF) μg/mL for Frac 8–IgG. Similarly, IgG from Frac 6– and Frac 8–immunized mice showed a neutralizing effect on HCVcc infection (Supplementary Figure 4). Thus, both fractions of the HCV particles appear to have similar immunogenicity. As a control, mice were immunized with naive Huh7-cultured media that was processed similar to the method used for the purification of HCV particles or saline together with adjuvant. IgG was purified from the mice sera, and HCVcc inhibition assay was performed. The purified IgG did not show a significant inhibitory effect for HCV infection compared with saline-immunized mice (Supplementary Figure 5). However, iHCV-IgG significantly inhibited the HCV infection at a concentration of 30 μg/mL. The effect of adsorption of the anti-E1 and anti-E2 antibodies in iHCV-IgG by incubation with recombinant J6E1 and J6E2/FLAG proteins on the inhibitory effect of iHCV-IgG then was examined. The HCV neutralizing effect of iHCV-IgG was reduced by adsorption of the anti-envelope antibodies, and the IC50 value was increased to 52.9 μg/mL (Figure 5*C*). These data indicate that anti-HCV envelope neutralizing antibodies were induced in mice by injection of the HCV-particle vaccine.

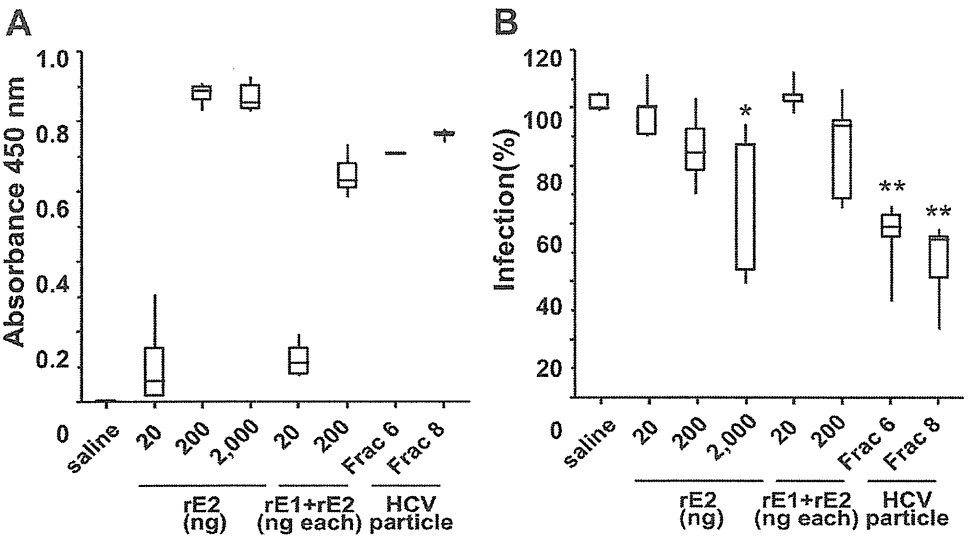

Figure 4. Neutralizing effects of sera from HCV-component and HCV-particle vaccine-immunized mice. BALB/c mice (n = 5) were immunized with recombinant proteins (20, 200, or 2000 ng of FLAG-tagged E2, 20 or 200 ng each of FLAG-tagged E1 and FLAG-tagged E2), or with purified HCV particles (2 pmol HCV core of Frac 6 or Frac 8) together with adjuvant. (*A*) The presence of anti-E2 antibodies in sera from immunized mice was analyzed using an enzyme immunoassay and the (*B*) ability of these sera to inhibit J6CF HCVpp infection of cultured Huh7.5.1 cells also was assayed. Each assay was performed in triplicate, and the data are presented as box plots. The statistical significance of differences between groups was analyzed using the Dunnett test (*$P < .05$, **$P < .001$ vs saline).

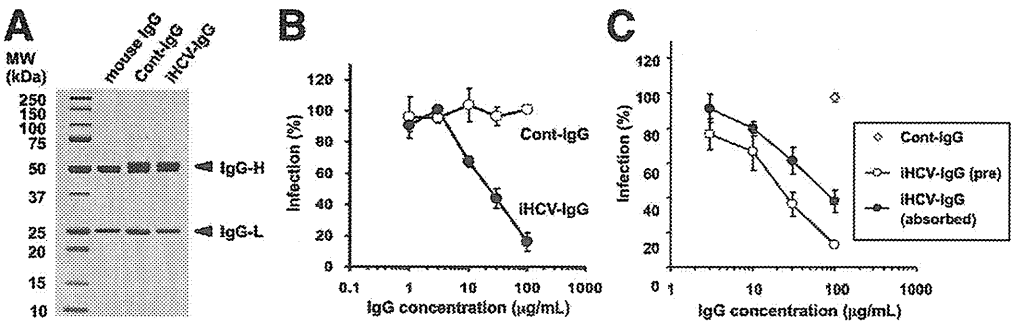

Figure 5. IgG purified from the sera of HCV-particle–immunized mice had a neutralizing effect against HCV infection in vitro. (*A*) IgG from the sera of mice at 7 weeks after immunization (iHCV-IgG) was purified using a KAPTIV-GY resin. Control mouse IgG was prepared similarly from saline-injected mice (Cont-IgG). Each IgG was analyzed for purity by SDS–polyacrylamide gel electrophoresis and Coomassie brilliant blue staining. As a control, a purchased mouse IgG was used. (*B*) Serially diluted Cont-IgG or iHCV-IgG was mixed with J6/JFH-1 HCVcc (multiplicity of infection, 0.1) and this mixture was inoculated into naive Huh7.5.1 cells. HCV infection was determined by analysis of the intracellular concentration of the HCV core protein. Infectivity is expressed as the percentage of cells that were infected. The IC50 value of iHCV-IgG was 16.1 μg/mL. (*C*) Anti-E1 and anti-E2 antibodies in iHCV-IgG were adsorbed by recombinant J6E1/FLAG and J6E2/FLAG protein, and the neutralizing effect of the E1E2-adsorbed IgG on HCV infection of Huh7 cells was assessed using the HCVcc inhibition assay as described for panel *B*. The IC50 of the E1E2-adsorbed IgG shifted to 52.9 μg/mL.

HCV Vaccine Induced Anti-HCV Infection In Vivo

As shown earlier, anti-HCV envelope neutralizing antibodies were induced in HCV-particle–immunized mice. To confirm the effect of the HCV-particle vaccine in vivo, we next examined its ability to neutralize an HCV challenge to human liver chimeric uPA-SCID mice. Purified IgG (100 μg) from HCV-particle–immunized mice was injected intraperitoneally into these chimeric mice 1 week before HCV infection. The mice then were challenged with multiple doses of 10^3, 10^4, and 10^5 RNA copies of J6/JFH-1 HCVcc at 2-week intervals. Blood samples were collected and HCV RNA was measured using RTD-PCR. HCV infection was inhibited (no infection) in all 6 mice in the vaccine-treated mouse group (iHCV-IgG) when 10^3 copies of HCV were inoculated (minimal infectious dose), whereas infection was observed in 4 of the 6 mice in the Cont-IgG mouse group. However, the neutralizing effect of iHCV-IgG vaccination was not detected when 10^4 or 10^5 copies of HCV were used for infection (Table 2). These data indicated that an inactivated HCV-particle vaccine potentially could be used as a prophylactic vaccine for HCV.

Discussion

There is no vaccine available for clinical use against HCV infection. Development of an HCV cell culture system has facilitated an understanding of the HCV life cycle, and the production of HCV prophylactic and therapeutic drugs. HCVcc mimics the native HCV particle, and thus has potential for use as an HCV-particle vaccine. In the present study, we established an efficient method for HCV particle production and purification, which allowed evaluation of the HCV particle as a vaccine candidate. HCV particles were purified from cell-cultured HCV of genotype 2a J6/JFH-1. The HCVcc in the culture supernatant was concentrated and partially purified by ultrafiltration using a 500-kilodalton cut-off membrane. Subsequently, HCVcc was purified by sucrose gradient ultracentrifugation. As reported previously,[12,13] the virus particles present in Frac 6 and Frac 8 after this sucrose gradient centrifugation displayed different characteristics. The virus in Frac 6 displayed low infectivity, but had a high concentration of viral components, whereas that in Frac 8 displayed high infectivity, but a low concentration of viral components. It is thought that the content of lipids or lipoproteins in these HCV particle fractions is different.[12,13] Finally, each viral fraction was purified and concentrated by ultrafiltration. When these HCV particles were inactivated, conjugated with a monophosphoryl lipid A plus trehalose-6,6-dimycolate adjuvant, and injected into mice, anti-E1 and anti-E2 antibodies were induced in the immunized mice, and the sera from these mice had a neutralizing effect against HCV infection in vitro. Because these sera displayed cross-reactivity for HCV strains 1a (H77), 1b (TH), and 2a (J6CF), it was considered that the HCV-particle vaccine had a broad neutralizing effect against HCV infection.

Western blot analysis of the envelope proteins in these HCV particles (Figure 3) indicated that the Frac 6 HCV particles contained approximately 10 ng each of the E1 and E2 proteins per 1 pmol HCV core protein. Because the molecular weight of the core, E1, and E2 proteins is approximately 20, 35, and 70 kilodaltons, respectively,[14] the molar ratio of the structural proteins in the purified HCV particles (core:E1:E2) was calculated to be 5:1:1 from Western blot analysis. Western blotting analysis also suggested that the amount of the E1 and E2 proteins in the Frac 8 HCV particle was likely to be higher than that

Table 2. Neutralizing Effect of IgG Purified From the Sera of HCV-Particle–Immunized Mice Against HCV Infection in Human Liver Chimeric uPA-SCID Mice

	Infected mice	
Virus challenge, HCV RNA copies	Cont-IgG	iHCV-IgG
10^3	4/6	0/6
10^4	5/6	6/6
10^5	6/6	6/6