strains isolated from human clinical samples at several of the inoculation doses. The results indicate that marmosets possess high sensitivity to DENV and could be a viable animal model for the evaluation of vaccine candidates and anti-DENV drugs.

In common marmosets, dengue vRNA was detected in lymphoid organs, lymph nodes, spleen, thymus and bone marrow, and also in non-lymphoid organs. DENV antigen-positive cells were detected in the spleen and liver of infected marmosets as in human patients (Bhamarapravati *et al.*, 1967; de Araújo *et al.*, 2009; Jessie *et al.*, 2004; Rosen *et al.*, 1999). Dengue vRNA was also detected in urine samples from DENV-infected marmosets (data not presented), as reported in human infection (Mizuno *et al.*, 2007; Poloni *et al.*, 2010). Of note, vRNA was detected in organs on day 14 when viraemia was no longer detected, as reported by other investigators (Marchette *et al.*, 1973). The results confirm propagation of DENV in inoculated marmosets.

Infection with one serotype of DENV induces life-long protective immunity to the same serotype in humans. Dengue vRNA and NS1 were not detected in plasma samples from marmosets that were reinoculated with DENV-2 at 33 weeks after primary inoculation (Table 1). The results indicate that protective immunity to the same serotype of DENV was induced by the primary infection as demonstrated in humans, and suggest that the marmoset model is potentially useful for evaluation of immunogenecity of candidate dengue vaccines. In contrast, using RT-PCR, viraemia was detected in all marmosets (D2-6, D2-7, D2-8 and D2-9) that were reinoculated with heterologous DENV serotypes (DENV-1 and DENV-3). Viraemia titres for D2-6 and D2-7 as assessed by RT-PCR were 5.1×10^4 copies ml^{-1} and 1.3×10^5 copies ml^{-1}, respectively, at day 7 post-inoculation with DENV-1, and viraemia titres for D2-8 and D2-9 were 3.8×10^5 copies ml^{-1} and 3.2×10^5 copies ml^{-1}, respectively, at day 7 post-inoculation with DENV-3 (M. L. Moi and others, unpublished data). Viraemia was not detected at day 7 after primary infection with DENV-1 and DENV-3 (Table 1). Although the results suggest that higher viraemia titres may have been induced in marmosets after secondary infection with heterologous DENV serotypes as compared with primary DENV infection, further studies using a greater number of marmosets are needed to address the clinical and pathophysiological aspects of heterologous secondary infection in marmosets. While other non-human primate models developed similar viraemia levels as marmosets shown in the present study (Guirakhoo *et al.*, 2004), the common marmoset model offers several advantages over other models for their use in research. These include small size (250–450 g), ease and lower cost of maintenance, availability and ease of handling which allows the introduction of flexibility into experimental procedures. Thus, common marmosets, in which high levels of viraemia were consistently detected upon DENV infection, could potentially provide a reliable and valuable model for evaluation of antiviral therapeutics and candidate vaccines against DENV.

METHODS

Cells. Vero cells were cultured in minimum essential medium (MEM; Sigma) with 10 % heat-inactivated FBS (Gibco) at 37 °C in 5 % CO_2. C6/36 cells were cultured in MEM with 10 % FBS and 1 % non-essential amino acids at 28 °C in 5 % CO_2.

Virus. DENV type 1 (DENV-1), 02-17/1 strain, DENV type 2 (DENV-2), DHF0663 strain (GenBank accession no. AB189122), D2/Hu/Jamaica/77/2007NIID (Jam/77/07) strain and D2/Hu/Maldives/77/2008NIID (Mal/77/08) strain, DENV type 3 (DENV-3), DSS1403 strain (GenBank accession no. AB189125) and DENV type 4 (DENV-4), 05-40/1 strain, were used for inoculation studies. The DENV-1 02-17/1 strain was isolated from an imported DF case from Indonesia. The DENV-2 DHF0663 strain was isolated from a DHF case in Indonesia. The DENV-2 Jam/77/07 and Mal/77/08 strains were isolated from imported DF cases from Jamaica and Maldives, respectively. The DENV-3 DSS1403 strain was isolated from a DSS case in Indonesia. The DENV-4 05-40/1 strain was isolated from an imported DF case from the Philippines. All DENV strains isolated from clinical samples were propagated with C6/36 cells and were used within four passages. Culture supernatant from infected C6/36 cells was centrifuged at 800 ***g*** for 5 min to remove cell debris, and then stored at −80 °C until use.

Infection of marmosets with DENV. A total of 20 male marmosets, weighing 258–512 g, were used. Marmosets were purchased from Clea Japan Inc. and caged singly at 27 ± 2 °C in 50 ± 10 % humidity with a 12 h light–dark cycle (lighting from 7:00 to 19:00) at Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Japan. Animals were fed twice a day with a standard marmoset diet (CMS-1M; CLEA Japan) supplemented with fruit, eggs and milk. Water was given *ad libitum*. The animals were in a healthy condition and confirmed to be negative for anti-DENV antibodies prior to primary DENV inoculation.

In experiment 1, the sensitivity of marmosets to each of the four serotypes of DENV was examined. Four marmosets were inoculated subcutaneously on the back with 3.5×10^7 p.f.u. of DENV-1 (02-17/1 strain), 6.7×10^7 p.f.u. of DENV-2 (DHF0663 strain), 4.5×10^6 p.f.u. of DENV-3 (DSS1403 strain) or 1.5×10^6 p.f.u. of DENV-4 (05-40/1 strain). Blood samples were collected before inoculation (day 0) and on days 3, 7, 10 and 14 after inoculation. Blood samples were used for the assessment of viraemia levels, IgG and IgM Abs, and the NS1 antigen level. All marmosets were euthanized on day 14 and organs were subjected to pathological examination.

In experiment 2, four marmosets were inoculated with DENV-2; two (D2-2 and D2-3) with 4.4×10^7 p.f.u. of DENV-2, DHF0663 strain, and two (D2-4 and D2-5) with 1.8×10^5 p.f.u. of DENV-2, DHF0663 strain. Blood samples were collected before inoculation (day 0) and on days 3, 7, 14 and 21 for the assessment of viraemia levels, IgG, IgM and the NS1 levels, and neutralizing Ab titres.

In experiment 3, four marmosets were used for the evaluation of their sensitivity to lower doses of DENV-2, DHF0663 strain; two (D2-6 and D2-7) with 1.8×10^4 p.f.u., and two (D2-8 and D2-9) with 1.8×10^3 p.f.u.

In experiment 4, four marmosets were inoculated with two other strains of DENV-2; two (D2-10 and D2-11) with 1.2×10^5 p.f.u. of Jam/77/07 strain, and two (D2-12 and D2-13) with 1.9×10^5 p.f.u. of Mal/77/08 strain. In these two experiments, blood samples were collected before inoculation (day 0) and on days 2, 4, 7, 14 and 21 for the assessment of viraemia levels, IgG, IgM and the NS1 levels.

In experiment 5, the distribution of DENV in various organs was examined. Four marmosets were inoculated with 6.7×10^7 p.f.u. of DENV-2, DHF0663 strain. One each was euthanized on days 3, 5, 8 or 14 after inoculation, and the organs collected for detection of dengue vRNA and histopathological analysis. Blood samples were collected from the animals before inoculation (day 0) and on days 2, 4, 7 and 13 for virus titration, IgG, IgM and the NS1 antigen detection.

In experiment 6, four marmosets used in experiment 2 were reinoculated with 1.8×10^5 p.f.u. of DENV-2, DHF0663 strain. Blood samples were collected on the day before inoculation (day 0) and on days 2, 4, 7, 15 and 21 for the assessment of viraemia levels and neutralizing Ab titres. Inoculation with DENV and blood drawing were performed under anaesthesia with 5 mg kg^{-1} of ketamine hydrochloride. Day 0 was defined as the day of virus inoculation.

All animal studies were conducted in accordance with 'Guides for animal experiments performed at National Institute of Infectious Diseases' approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases, Japan (approval nos 608011 and 609014), and 'National Institute of Biomedical Innovation rules and guidelines for experimental animal welfare' approved by the National Institute of Biomedical Innovation, Japan (approval nos 20-003 and 21-013).

Titration of vRNA in plasma and organs. Plasma samples were stored at −80 °C until use. Collected organs were homogenized by BioMasher (Nippi). Homogenized samples were suspended with 0.5 ml PBS(−) and centrifuged at 800 *g* for 5 min. After centrifugation, supernatants were collected and stored at −80 °C until use. vRNA was isolated from plasma and organ samples, using the High Pure Viral RNA kit (Roche Diagnostics). As housekeeping genes vary among tissues, experimental treatment and infection, total RNA was used to avoid specific expression profiles among housekeeping genes. Levels of dengue vRNA were determined by quantitative real-time RT-PCR as previously reported (Ito *et al.*, 2004). All RT-PCR assays were performed in duplicate.

Detection of DENV NS1, anti-DENV IgM and IgG Abs in plasma. Levels of NS1 in plasma samples were assessed by Platelia Dengue NS1 Ag assay (Bio-Rad). The assay was performed according to the manufacturer's instructions with modification to the applied volume of plasma. ELISA index was calculated by the formula: optical density (OD) of the test sample divided by the mean OD of the cut-off control (tested in duplicate). ELISA indexes of <1 and $\geqslant 1$ were considered to be negative and positive, respectively.

DENV-specific IgM Ab was detected by Dengue Fever IgM Capture ELISA (Focus) and DENV-specific IgG Ab was examined by Dengue IgG Indirect ELISA (PanBio) according to the manufacturer's instructions. The positive/negative (P/N) ratio was calculated by the formula: OD of the test sample divided by the OD of a negative sample. Plasma samples collected on the day of inoculation were used as the negative samples. P/N ratios of <2 and $\geqslant 2$ were considered to be negative and positive, respectively. All ELISAs were conducted in duplicates (Moi *et al.*, 2010a).

Titration of neutralizing Abs to DENV-2. Neutralizing Ab titres were determined by using plaque reduction neutralization tests with DENV-2, DHF0663 strain. Heat-inactivated serum samples were serially diluted twofold from 1:20 to 1:1250 with MEM supplemented with 2% FCS. Virus–Ab mixture was prepared by mixing 50 µl DENV-2 at titres of 1000 p.f.u. ml^{-1} with 50 µl of serially diluted serum samples. Control virus samples were prepared by mixing 50 µl DENV-2 at titres of 1000 p.f.u. ml^{-1} with 50 µl MEM supplemented with 2% FCS. Virus–Ab mixture was incubated at 37 °C for 1 h. One hundred microlitres of virus–Ab mixture was inoculated onto Vero monolayers into six-well plates. After 5 days of inoculation, cells were fixed and stained, and plaques were counted. Neutralization titre is expressed as the maximum dilution of plasma sample that yielded a >50% plaque reduction in the virus inoculum as compared with control virus sample (Moi *et al.*, 2010b).

Immunohistochemical analysis. The livers and spleens from marmosets were fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin-embedded tissues were cut into 4 µm sections. For immunohistochemical analysis, a series of sections was stained for DENV antigen using a marmoset polyclonal anti-DENV Ab conjugated with horseradish peroxidase. Sections were counterstained with haematoxylin.

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Long-term persistent GBV-B infection and development of a chronic and progressive hepatitis C-like disease in marmosets

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It has been shown that infection of GB virus B (GBV-B), which is closely related to hepatitis C virus, develops acute self-resolving hepatitis in tamarins. In this study we sought to examine longitudinally the dynamics of viral and immunological status following GBV-B infection of marmosets and tamarins. Surprisingly, two of four marmosets but not tamarins experimentally challenged with GBV-B developed long-term chronic infection with fluctuated viremia, recurrent increase of alanine aminotransferase and plateaued titers of the antiviral antibodies, which was comparable to chronic hepatitis C in humans. Moreover, one of the chronically infected marmosets developed an acute exacerbation of chronic hepatitis as revealed by biochemical, histological, and immunopathological analyses. Of note, periodical analyses of the viral genomes in these marmosets indicated frequent and selective non-synonymous mutations, suggesting efficient evasion of the virus from antiviral immune pressure. These results demonstrated for the first time that GBV-B could induce chronic hepatitis C-like disease in marmosets and that the outcome of the viral infection and disease progression may depend on the differences between species and individuals.

Keywords: GBV-B, HCV, marmoset, tamarin, hepatitis C

INTRODUCTION

Among the known viruses, GB virus B (GBV-B) is closely related to hepatitis C virus (HCV), with 25–30% homology at the amino acid level, and is tentatively classified in *Hepacivirus* genus of *Flavivirus* family (Muerhoff et al., 1995; Simons et al., 1995; Ohba et al., 1996). Due to limited epidemiological analyses, the natural host(s) and prevalence of GBV-B have remained to be determined.

Hepatitis C virus is a major causative agent for non-A, non-B hepatitis. HCV is globally disseminated and estimated to be carried by more than 170 million people (Chisari, 2005; Lavanchy, 2009). Most HCV-infected individuals develop chronic liver diseases such as liver cirrhosis and hepatocellular carcinoma (Hoofnagle, 1997; Seeff and Hoofnagle, 2002; Rehermann and Nascimbeni, 2005). Since standard therapy with PEGylated interferon and ribavirin is effective for only about 50% of patients, it is crucial to develop more effective therapeutics (Feld and Hoofnagle, 2005; Melnikova, 2008). The only validated animal model for HCV infection is the chimpanzees. This model has been valuable for determining important aspects of this disease, including the relationship between the virus and the antiviral immune responses of the host and the process of viral pathogenesis (Bukh, 2004; Akari et al., 2009; Boonstra et al., 2009). However, chimpanzees are endangered and present ethical complications and the availability of these experimental animals is severely restricted.

When tamarins (members of the New World monkeys) are infected with GBV-B, they generally develop acute viremia and self-resolving hepatitis as indicated by increases in the levels of serum enzymes such as alanine aminotransferase (ALT) (Bukh et al., 1999; Beames et al., 2000; Beames et al., 2001; Sbardellati et al., 2001; Lanford et al., 2003; Martin et al., 2003; Bright et al., 2004; Jacob et al., 2004; Nam et al., 2004; Kyuregyan et al., 2005; Ishii et al., 2007; Weatherford et al., 2009). Thus, the monkeys have been proposed as a surrogate model of HCV infection of chimpanzee and humans. However, a major hurdle for the development of a monkey-based surrogate model is the difficulties encountered in obtaining chronically infected monkeys that exhibit progression of chronic hepatitis C-like diseases (Martin et al., 2003; Nam et al., 2004; Takikawa et al., 2010).

Abbreviations: ALT, alanine aminotransferase; GBV-B, GB virus B; HCV, hepatitis C virus; HE, hematoxylin and eosin; p.i., post infection.

It has recently been shown that marmosets, another member of New World monkeys, are susceptible to GBV-B infection and develop relatively lower levels of acute viremia (10^5–10^8 copies/ml) as compared with that in tamarins (10^7–10^{10} copies/ml) (Lanford et al., 2003; Bright et al., 2004; Woollard et al., 2008; Weatherford et al., 2009), although it remains elusive whether the marmosets could permit persistent GBV-B infection. Considering that the viral loads in the acute phase of experimental HCV infection of chimpanzees that consequently develop persistent infection are generally 10^7 copies/ml or less (Fernandez et al., 2004; Bukh et al., 2008), it is possible that the lower viral loads in the acute phase is preferable for the establishment of viral persistency. We thus initiated studies of the dynamics of viral and immunological status following GBV-B infection of tamarins and marmosets in a longitudinal follow-up study. We show here for the first time that GBV-B infection produces a chronic and progressive hepatitis C-like disease in marmosets as demonstrated by fibrosis and a recurrent ALT increase and that one of the marmosets experienced acute exacerbation of chronic hepatitis as indicated by piecemeal necrosis and an ALT flare >4 years after infection.

MATERIALS AND METHODS

ANIMALS

Adult red-handed tamarins (*Saguinus midas*) and common marmosets (*Callithrix jacchus*) were housed in individual cages at the Tsukuba Primate Research Center. All animal studies were conducted in accordance with the protocols of experimental procedures that were approved by the Animal Welfare and Animal Care Committees of the National Institute of Biomedical Innovation and the National Institute of Infectious Diseases.

GBV-B INFECTION IN TAMARINS AND MARMOSETS

GBV-B infectious serum obtained from a tamarin (1.3×10^9 viral RNA copies per inoculum) was injected into each tamarin and marmoset intrahepatically as previously described (Ishii et al., 2007). We confirmed that the inoculum contained no mutations as compared with the original sequence. Of note, an anti-luciferase siRNA in a cationic liposome formulation was administered to one of the marmosets (Cj05-002) 2 days before the infection, which was performed as previously described (Yokota et al., 2007). Blood samples were periodically collected from the femoral vein of each animal under anesthesia and the plasma samples were evaluated for GBV-B genomic RNA, ALT, and antibodies against GBV-B core and NS3 proteins.

QUANTIFICATION OF GBV-B GENOMIC RNA

GBV-B RNA was isolated from the plasma samples by using a QIAamp MinElute Virus Spin kit (QIAGEN) and was quantified by real-time PCR using the 5′-exonuclease PCR (TaqMan) assay system (Ishii et al., 2007). The primers 558F [5′-AACGAGCAAAGCGCAAAGTC] and 626R [5′-CATCATGGATACCAGCAATTTTGT] and the probe 579P [5′-FAM-AGCGCGATGCTCGGCCTCGTA-TAMRA] (Beames et al., 2000) were obtained from Sigma-Aldrich. The cutoff value was 10^3 copies/ml. All the specimens were evaluated in duplicate and the average values were calculated.

DETECTION OF ANTIBODIES AGAINST GBV-B CORE AND NS3 PROTEINS BY ELISA

Tamarin and marmoset plasma samples were evaluated for anti-GBV-B core and NS3 antibodies by ELISA as described previously (Ishii et al., 2007).

HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSES

Liver samples obtained by necropsy from the GBV-B-infected marmoset were examined histopathologically as previously described (Ishii et al., 2007). For standard histological examination, the sections were subjected to hematoxylin and eosin (HE) staining. Masson's trichrome staining was also performed to estimate the development of fibrosis according to a standard laboratory protocol. To detect the viral protein in tissues, we employed a mouse anti-core monoclonal antibody, 5A10, that we generated. In brief, Mice were immunized with the GBV-B core protein expressed in *E. coli* (Ishii et al., 2007). Hybridoma cells producing an anti-core mAb were screened by both the core-expressing 293T cells and the liver sections of an acutely GBV-B-infected tamarin. Liver samples were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections were deparaffinized by pretreating with 0.5% periodic acid and then subjected to antigen retrieval with citric acid buffer and heating in an autoclave for 10 min at 121°C. The sections were then incubated free floating in primary antibody solution (5A10; 1:50 dilution) overnight at 4°C. Following brief washes with wash buffer, the sections were sequentially incubated with a biotinylated goat anti-mouse IgG (1:400 dilution), followed by addition of a streptavidin–biotin–horseradish peroxidase complex (sABC kit; DAKO, Denmark). Immunoreactive elements in the sections were visualized by treatment with 3,3′-diaminobenzidine tetroxide (Dojin Kagaku, Japan), together with counterstaining with hematoxylin.

DETERMINATION OF THE GBV-B SEQUENCE

Viral RNA was isolated from the plasma of GBV-B-infected marmosets as described above. GBV-B cDNA was synthesized using SuperScript reverse transcriptase III (Invitrogen) with random hexamer primers (Invitrogen). The resulting cDNAs were used to obtain PCR amplification products of lengths of 0.5–1.0 kb, using GBV-B-specific primers and LA-Taq DNA polymerase (TaKaRa). The PCR products were then purified from the gel using a QIA-quick gel extraction kit (QIAGEN), and the purified amplimers were sequenced directly using a CEQ-2000XL analysis system (Beckman) with a DTCS quick start kit and GBV-B-specific primers according to the manufacturer's instructions. Sequence data were analyzed using the Sequencher 4.8 (Gene Codes) and Mac Vector 10.6 (MacVector) software packages. The GenBank accession numbers of the viral genome sequences in each time point are as follows: AB630358, AB630359, and AB630360 for 45, 104, and 135 weeks after infection in Cj05-002; AB630361, AB630362, AB630363, and AB630364 for 33, 88, 141, and 229 weeks after infection in Cj05-004, respectively. Throughout this article, the amino acids are numbered according to the full-length genome sequence of isolate pGBB (GenBank accession number AF179612).

RESULTS

GBV-B INFECTION IN TAMARINS AND MARMOSETS

Four tamarins and four marmosets were intrahepatically inoculated with GBV-B and the growth kinetics and pathogenesis of the virus were compared. In tamarins, the peak viral loads in plasma reached 10^9–10^{10} copies/ml in the acute phase and the viremia was maintained for an average of 3 months in parallel with increases in plasma ALT levels (**Figure 1A**). Antibodies reactive with the viral core and NS3 proteins were developed in all of the tamarins as the plasma viral loads were reduced and the antibody titers reached maximum levels concurrently with the complete loss of detectable viral RNA (**Figure 1A**). In contrast, two of four marmosets infected with GBV-B developed chronic infection while the others exhibited a phenotype similar to that of the tamarins (i.e., subacute clearance of the viremia followed by antibody responses). One exception is that lower plasma viral loads (10^7–10^8 copies/ml) were observed in the marmosets relative to those of the tamarins (**Figure 1B**). The details of the chronically infected marmosets are described below.

Case 1: Cj05-002 (**Figures 1B** and **2**). The viral RNA was undetectable until week 4 post infection (p.i.) and then gradually increased to a peak at week 18 p.i. (3×10^7 copies/ml). Subsequently, this case retained intermittent viremia during the observation period of week 180 p.i., while the intervals between the viremia phases were prolonged. Importantly, the titers of anti-core and anti-NS3 antibodies reached a persistent plateau at 6 months and 1 year p.i., respectively. In addition, ALT levels were recurrently increased without observation of other clinical symptoms.

Case 2: Cj05-004 (**Figures 1B** and **2**). During the acute phase of infection, the level of viremia was relatively low and transient, followed by a 1-year period when the virus was essentially undetectable. Irrespective of the very low viral load, the titer of anti-NS3 but not anti-core antibody steadily increased and reached a plateau at week 42 p.i. Moreover, an occasional but obvious increase in the level of ALT was observed during this period. We thus suspected that antigenic stimulation by a lower level of viral growth in the liver, which remained below detectable levels in blood, might lead to the induction of the anti-NS3 antibody and the recurrent ALT increase. Subsequently, viremia became detectable at week 58 p.i. and 10^4–10^5 copies/ml of the viral RNA persisted until week 108 p.i. Thereafter, an abrupt increase of the anti-core antibody was detected, concomitant with augmentation of the viral load of $10^{5.5}$ copies/ml on average and recurrent increases in the ALT level. Eventually, the individual was euthanized at week 229 p.i. because of poor prognosis since the ALT value drastically increased by 161-fold, which was accompanied by a dramatic decrease of platelet counts and a deteriorating general status. Histopathological analyses of the necropsy samples demonstrated that the liver developed diffuse piecemeal necrosis with infiltration of lymphocytes and

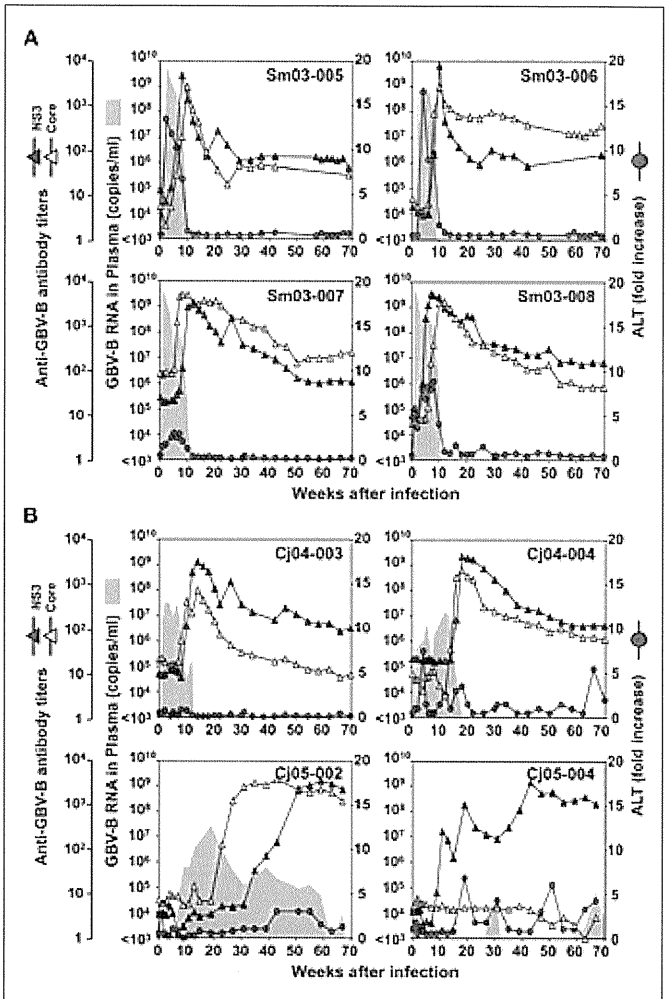

FIGURE 1 | Infection profiles until week 70 p.i. of tamarins (A) and marmosets (B) inoculated intrahepatically with GBV-B infectious serum obtained from a tamarin. Plasma samples periodically obtained from the monkeys were evaluated for the copy numbers of GBV-B genomic RNA (green shaded area), ALT levels (red circles), and the antibody titers against GBV-B core and NS3 (blue and yellow triangles, respectively).

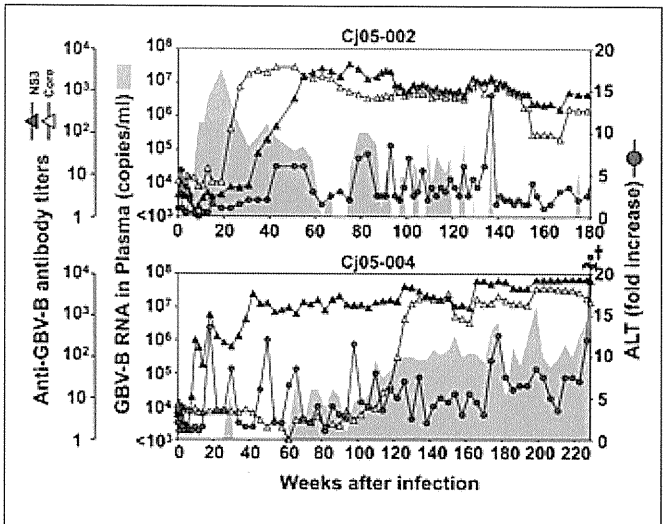

FIGURE 2 | Profiles of the marmosets persistently infected with GBV-B. Plasma samples periodically obtained from the marmosets were evaluated for the copy numbers of GBV-B genomic RNA (green shaded area), ALT levels (red circles), and the antibody titers against GBV-B core and NS3 (blue and yellow triangles, respectively). The cruciate mark in Cj05-004 indicates that the individual was euthanized due to a poor prognosis at week 229 p.i. At that time, the ALT value in plasma had increased by 161-fold.

formation of lymphoid follicles (**Figure 3A**, Appendix). The viral load in the liver was relatively high (3.8×10^4 copies/mg tissue weight), which was similar to the viral load observed for tamarins acutely infected with GBV-B (Ishii et al., 2007). The high viral load in the liver was consistent with a large number of granular positive signals for the core protein, which was in similar manner with the core protein of HCV (Miyanari et al., 2007), as immunostained with an anti-GBV-B core monoclonal antibody (**Figure 3B**). Notably, Masson trichrome staining (**Figures 3C,D**) as well as Elastica van Gieson staining (Appendix) demonstrated that the liver also developed diffuse and abundant fibrosis. The disease of this marmoset was therefore diagnosed as a case of acute exacerbation of progressive chronic hepatitis by GBV-B infection.

ANALYSIS OF MUTATIONS IN GBV-B GENOMES

Next, we determined the dominant sequence of the viral genomes at weeks 45, 104, and 135 p.i. in Cj05-002 and weeks 33, 88, 141, and 229 p.i. in Cj05-004. As seen in **Figure 4A**, it was found that there was no specific region in which extensive nucleotide mutations occurred throughout the study periods and that the nucleotide mutation rates were $1.9–2.9 \times 10^{-3}$ and $1.5–3.6 \times 10^{-3}$ changes per site per year in Cj05-002 and Cj05-004, respectively (**Table 1**). In terms of amino acid substitution, we observed the following: (i) several back or sequential mutations (G250V > A, S731L > S, E2346G > E in Cj05-002; V254A > V, I285V > I, L495S > L, T735A > T, F2135L > F > S in Cj05-004) in both marmosets; (ii) highly selective non-synonymous mutations that were remarkable in E1, but such mutations were rarely observed in core (**Figures 4** and **5**); and (iii) the non-synonymous mutation rates were $1.8–4.0 \times 10^{-3}$ and $2.1–4.6 \times 10^{-3}$ substitutions per site per year in Cj05-002 and Cj05-004, respectively (**Figures 4** and **5**; **Table 2**). (iv) The non-synonymous changes detected mainly in NS5A and NS5B in both animals were also observed in a number of previous reports (Simons et al., 1995; Bukh et al., 1999; Sbardellati et al., 2001; Martin et al., 2003; Nam et al., 2004; Kyuregyan et al., 2005; Weatherford et al., 2009; Takikawa et al., 2010). It may be reasonable to consider that the molecular clone we employed (Bukh et al., 1999) was derived from a minor clone of mixed populations and emergence of a new mutations easily occurred as a mechanism of GBV-B adaptation to a new host, while it is also possible that "consensus" non-synonymous changes were due to either a result of a selection of the pre-existent minor variants. Taken together, these results suggest that efficient

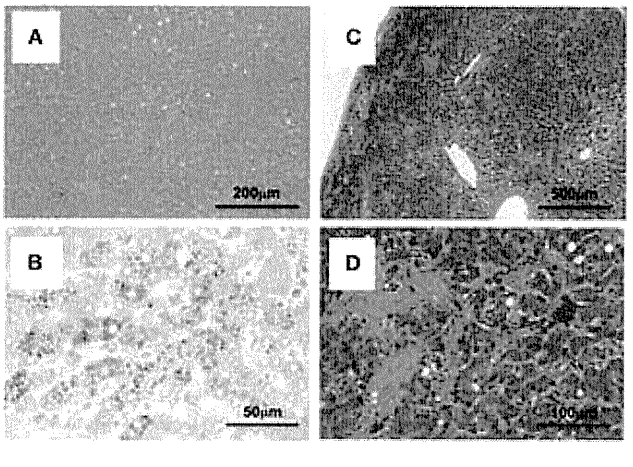

FIGURE 3 | Histopathological and immunohistochemical analyses of the liver from Cj05-004 at week 229 p.i. HE staining (A), immunohistochemical staining for the core protein of GBV-B (B), and Masson's trichrome staining (C,D) are shown.

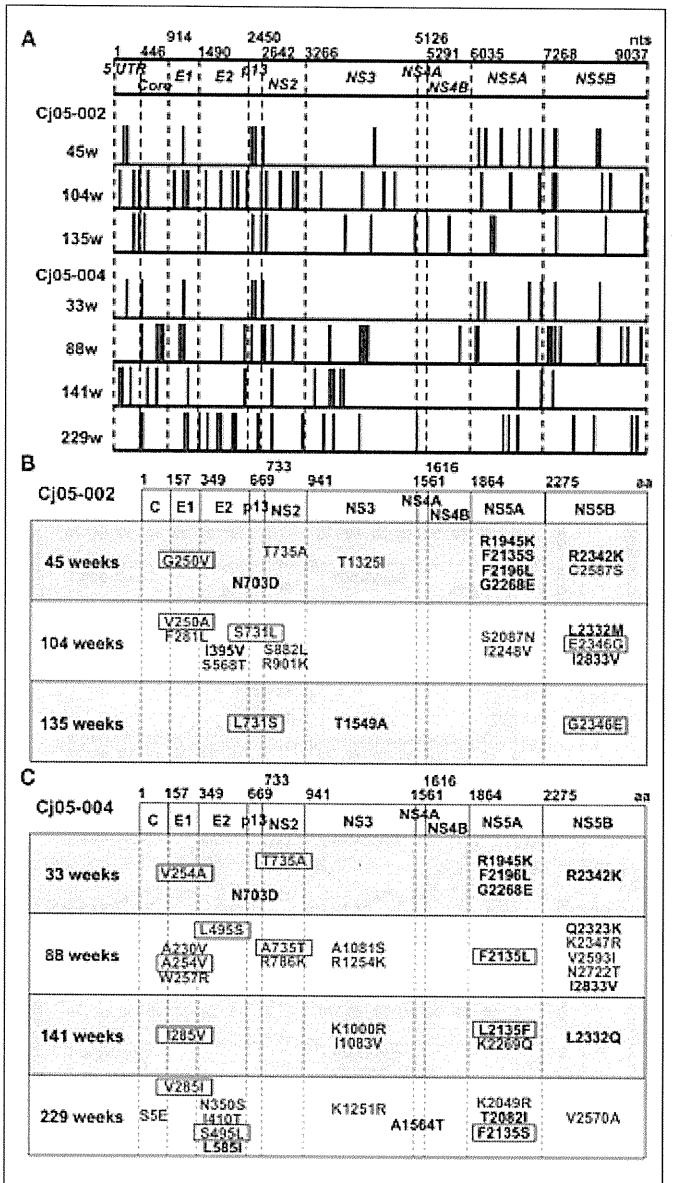

FIGURE 4 | Mutations in the viral genome sequences amplified from plasma of the marmosets persistently infected with GBV-B. (A) Positions of the nucleotide mutations in the viral genome sequences at multiple time points (at weeks 45, 104, and 135 in Cj05-002 and weeks 33, 88, 141, and 229 in Cj05-004) are illustrated as bars. **(B,C)** Positions of the non-synonymous mutations in the viral genome sequences at multiple time points are shown. **(B)** Cj05-002; **(C)** Cj05-004. Positions of the mutations that had been identified in previous reports are indicated as black, while those unidentified previously are shown as blue. Red squares illustrate back or sequential mutations.

Table 1 | Summary of the nucleotide substitutions in GBV-B genome sequences amplified from plasma of the marmosets persistently infected with GBV-B.

Genomic region	nt position	No. (%) of nt differences						
		Cj05-002			Cj05-004			
		45 weeks	104 weeks	135 weeks	33 weeks	88 weeks	141 weeks	229 weeks
5′UTR	1–445	2 (0.45)	3 (0.67)	2 (0.45)	1 (0.22)	0 (0)	3 (0.67)	0 (0)
Core	446–913	0 (0)	1 (0.21)	1 (0.21)	1 (0.21)	4 (0.85)	2 (0.43)	3 (0.64)
E1	914–1489	1 (0.17)	3 (0.52)	0 (0)	1 (0.17)	3 (0.52)	1 (0.17)	2 (0.35)
E2	1490–2449	0 (0)	5 (0.52)	1 (0.10)	0 (0)	2 (0.21)	1 (0.10)	6 (0.63)
p13	2450–2641	2 (1.04)	1 (0.52)	2 (1.04)	2 (1.04)	1 (0.52)	0 (0)	1 (0.52)
NS2	2642–3265	1 (0.16)	5 (0.80)	1 (0.16)	1 (0.16)	4 (0.64)	1 (0.16)	2 (0.32)
NS3	3266–5125	1 (0.05)	4 (0.22)	3 (0.16)	0 (0)	5 (0.27)	6 (0.32)	3 (0.16)
NS4A	5126–5290	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.61)
NS4B	5291–6034	0 (0)	0 (0)	2 (0.27)	0 (0)	1 (0.13)	0 (0)	0 (0)
NS5A	6035–7267	6 (0.49)	4 (0.32)	2 (0.16)	4 (0.32)	4 (0.32)	2 (0.16)	3 (0.24)
NS5B	7268–9037	4 (0.23)	5 (0.28)	3 (0.17)	2 (0.11)	10 (0.56)	1 (0.06)	4 (0.23)
Total	9037	17 (0.19)	31 (0.34)	17 (0.19)	12 (0.13)	34 (0.38)	17 (0.19)	25 (0.28)
Mutation rate/year		2.2×10^{-3}	3.0×10^{-3}	3.2×10^{-3}	2.1×10^{-3}	3.6×10^{-3}	1.8×10^{-3}	1.6×10^{-3}

Table 2 | Summary of the amino acid substitutions in GBV-B genome sequences amplified from plasma of the marmosets persistently infected with GBV-B.

Amino acid region	aa position	No. (%) of aa differences						
		Cj05-002			Cj05-004			
		45 weeks	104 weeks	135 weeks	33 weeks	88 weeks	141 weeks	229 weeks
Core	1–156	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.64)
E1	157–348	1 (0.52)	2 (1.04)	0 (0)	1 (0.52)	3 (1.56)	1 (0.52)	1 (0.52)
E2	349–613	0 (0)	2 (0.63)	0 (0)	0 (0)	1 (0.31)	0 (0)	4 (1.25)
P13	669–732	1 (1.56)	1 (1.56)	1 (1.56)	1 (1.56)	0 (0)	0 (0)	0 (0)
NS2	733–940	1 (0.48)	2 (0.96)	0 (0)	1 (0.48)	2 (0.96)	0 (0)	0 (0)
NS3	941–1560	1 (0.16)	0 (0)	1 (0.16)	0 (0)	2 (0.32)	2 (0.32)	1 (0.16)
NS4A	1561–1615	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1.82)
NS4B	1616–1863	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
NS5A	1864–2274	4 (0.97)	2 (0.49)	0 (0)	3 (0.73)	1 (0.24)	2 (0.49)	3 (0.73)
NS5B	2275–2864	2 (0.34)	3 (0.51)	1 (0.17)	1 (0.17)	5 (0.85)	1 (0.17)	1 (0.17)
Total	2864	10 (0.38)	12 (0.42)	3 (0.10)	7 (0.24)	14 (0.49)	6 (0.21)	12 (0.42)
Mutation rate/year		4.0×10^{-3}	3.7×10^{-3}	1.8×10^{-3}	3.9×10^{-3}	4.6×10^{-3}	2.1×10^{-3}	2.5×10^{-3}

and selective evasion from immune pressure in the two marmosets resulted in long-term persistent GBV-B infection accompanied by subsequent chronic hepatitis.

DISCUSSION

In this study, we show for the first time that GBV-B is capable of eliciting a chronic and progressive hepatitis C-like disease in marmosets. Evidence for this condition is demonstrated by long-term persistent GBV-B infection, recurrent ALT increase, and fibrosis. Moreover, one of the chronically infected marmosets developed acute exacerbation of chronic hepatitis as indicated by diffuse piecemeal liver necrosis and an ALT flare, which is seen in patients with viral hepatitis (Perrillo, 1997). While the usefulness of the monkey model as a surrogate model for HCV infection has been under debate due to the virtual inability of GBV-B to cause chronic hepatitis C-like disease in tamarins, the present data demonstrate that the ability of GBV-B to induce the chronic disease is likely to be inherent depending on the differences between species and individuals.

It has been reported that tamarins generally permit extensive replication of GBV-B in the subacute phase of infection and develop acute hepatitis as shown by significant increases of serum enzymes such as ALT and isocitrate dehydrogenase. The viral load in marmosets seems to be lower than in tamarins (Lanford et al.,

2003; Bright et al., 2004; Woollard et al., 2008; Weatherford et al., 2009). A recent report indicated that marmosets exhibit susceptible and partially resistant phenotypes upon infection with GBV-B (Weatherford et al., 2009). Consistent with this finding, the present results also showed that the marmosets appeared to exhibit two phenotypes (**Figure 1B**). Importantly, the long-term persistent GBV-B infection was established in the marmosets with lower viral loads during the initial weeks p.i. (**Figure 1B**; Cj05-002 and Cj05-004). This suggests that the mild viral growth in the marmosets with a "partially resistant" phenotype is critical for the establishment of the chronic infection. Of note, the viral growth was undetectable until week 6 p.i. in Cj05-002, owing to unexpected interferon responses that were induced by administration of an anti-luciferase small interfering RNA in a cationic liposome formulation 2 days before GBV-B infection (Yokota et al., 2007). Irrespective of the partial suppression of the viral growth, humoral immune responses were delayed and consequently the individual developed chronic infection. Taken together, it is reasonable to assume that the viral persistence in marmosets may be closely associated with inefficient antiviral immune responses that are elicited at the periods of the lower viral loads. Previously, we and others employed relatively higher amounts of GBV-B for challenge in tamarins and marmosets. This could result in greater viral loads in the acute phase than those in humans and chimpanzees infected with HCV, followed by induction of efficient protective immunity and acute clearance. To clarify the mechanisms by which chronic GBV-B infection is established, further characterization of the differences in innate and acquired antiviral immunity between individuals with acute clearance and chronic infection will be needed.

Accumulating evidence suggests that escape mutations occurring during the course of chronic HCV infection may lead to evasion of humoral and cellular antiviral immunity (Bowen and Walker, 2005a,b; Burke and Cox, 2010). Consistent with these observations, we found that GBV-B acquired multiple back or sequential non-synonymous mutations (e.g., G250V > A, S731L > S, E2346G > E in Cj05-002; and V254A > V, I285V > I, L495S > L, T735A > T, F2135L > F > S in Cj05-004) in the chronically infected marmosets. Highly selective non-synonymous mutations were identified especially in E1, but such mutations were rarely observed in core (**Figures 4** and **5**). Moreover, the non-synonymous mutations in the E1 and NS3 regions occurred throughout the observation periods in Cj05-004 with chronic GBV-B infection, which had not been identified previously (Simons et al., 1995; Bukh et al., 1999; Sbardellati et al., 2001; Martin et al., 2003; Nam et al., 2004; Kyuregyan et al., 2005; Weatherford et al., 2009; Takikawa et al., 2010). Together with the finding that the rates of both synonymous and non-synonymous mutations were similar to those observed in cases of HCV (Ogata et al., 1991; Fernandez et al., 2004), these results strongly suggest that efficient and selective evasion from immune pressures may result in long-term persistent GBV-B infection and subsequent chronic hepatitis. Further analyses on the functional significance of the non-synonymous mutations will clarify this possibility.

It is surprising that in Cj05-004, the antibody titer to NS3 was observed to steadily increase after week 10 p.i. irrespective of the scarce viral loads over 1 year p.i., including the bipartite

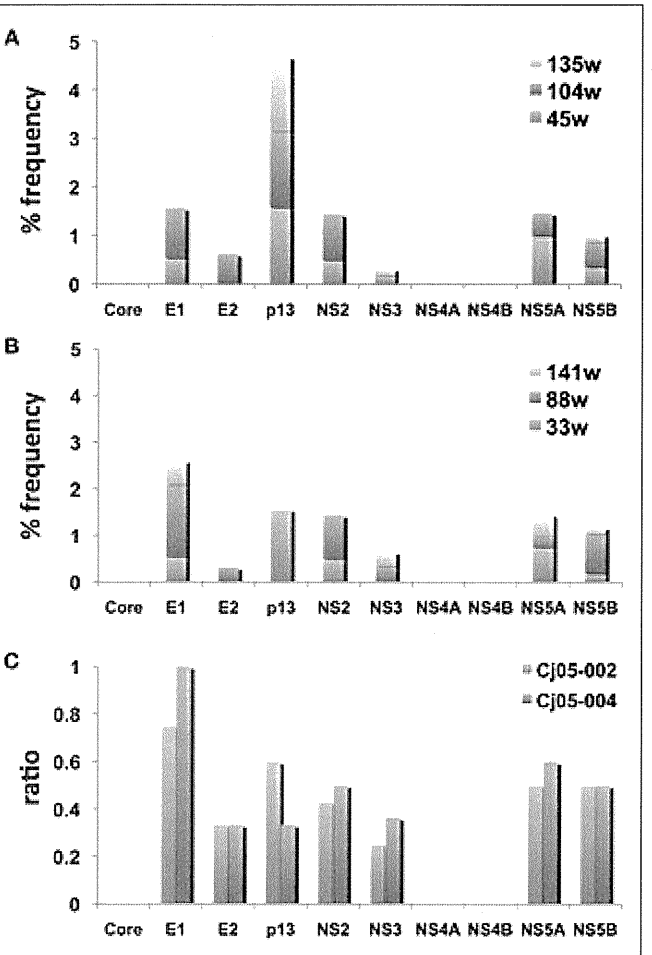

FIGURE 5 | The frequency of the non-synonymous mutations in the viral genome sequences amplified from plasma of the marmosets persistently infected with GBV-B. (A,B) The accumulated frequency of the non-synonymous mutations per total amino acid numbers of each viral protein at multiple time points are illustrated [**(A)** at weeks 45, 104, and 135 in Cj05-002; **(B)** weeks 33, 88, and 141 in Cj05-004]. To compare the frequency between the two marmosets, the data at week 229 p.i. in Cj05-004 are omitted. **(C)** The ratio of the non-synonymous mutations per total numbers of nucleotide mutations identified at weeks 45, 104, and 135 in Cj05-002 and at weeks 33, 88, and 141 in Cj05-004 in each viral gene. To compare the frequency between the two marmosets, the data at week 229 p.i. in Cj05-004 are omitted.

periods of weeks 4–26 and 34–58 p.i. when the virus was undetectable (**Figure 1**). Considering that three spikes of ALT levels were observed during these periods, our results suggest that antigenic stimulation by the lower level of viral growth in the liver, which was below detectable levels in blood, may induce the antibody and cytotoxic T-cell responses. In addition, during longitudinal analyses of monkeys experimentally infected with GBV-B, it is important to comprehensively evaluate multiple parameters, including viral loads, serum enzymes, and antibodies against core and NS3 proteins, to define whether virus-infected monkeys that produce no detectable viremia for a period of time have cleared the virus or are experiencing a latent period of chronic infection.

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APPENDIX

MATERIALS AND METHODS

Liver samples obtained by necropsy from the GBV-B-infected marmosets were histopathologically analyzed as described in Section "Materials and Methods." Elastica–van Gieson staining was performed to evaluate fibrosis according to a standard laboratory protocol. To detect CD3 and CD20 antigens, liver samples were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections were deparaffinized by pretreatment with 0.5% periodic acid and then subjected to antigen retrieval with citric acid buffer and heating in an autoclave for 10 min at 121°C. Sections were then incubated free floating in the monoclonal antibody solution for CD20 (DAKO) and CD3 (DAKO) overnight at 4°C. Following brief washes with buffer, the sections were sequentially incubated with biotinylated goat anti-mouse IgG (1:400), followed by streptavidin–biotin–horseradish peroxidase complex (sABC kit; DAKO, Denmark). Immunoreactive elements were visualized by treating the sections with 3,3′-diaminobenzidine tetroxide (Dojin Kagaku, Japan). The sections were then counterstained with hematoxylin.

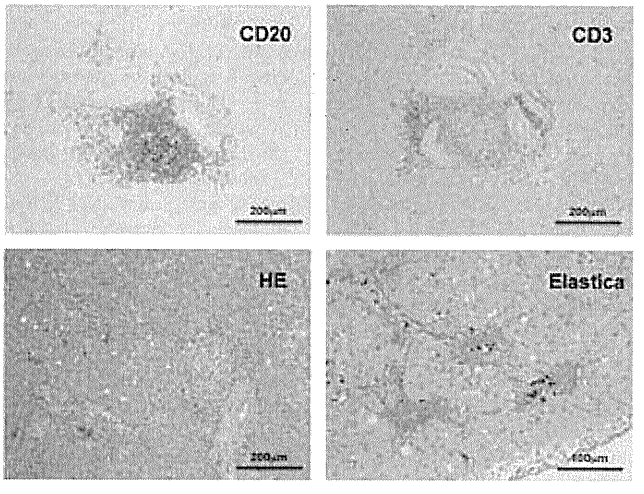

FIGURE A1 | Histopathological and immunohistochemical analyses of the liver from Cj05-004 at week 229 p.i.

13

Macaque-Tropic HIV-1 Derivatives: A Novel Experimental Approach to Understand Viral Replication and Evolution In Vivo

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1. Introduction

The use of animal models in the study of human diseases is obviously important. Fundamental properties of the disease can be investigated analytically and thoroughly by this approach, contributing much to the progress of basic science as well as clinical medicine (Nomaguchi & Adachi, 2010). Researchers in various specialties, therefore, have made every effort to establish animal models for human diseases including those caused by infectious agents. Acquired immunodeficiency syndrome (AIDS) of humans has long been one of the major targets for the model study in appropriate animals. However, human immunodeficiency virus type 1 (HIV-1) itself, the predominant causative virus of human AIDS, can not be used due to its very narrow host range. Because HIV-1 has adapted itself dexterously from the ancestral virus to replicate, persist and spread strictly in humans, it is very unique among various primate immunodeficiency viruses and no good counterparts are available in nature (Desrosiers, 2007; Kirchhoff, 2009; Sauter et al., 2009). Therefore, it can be concluded that practical and meaningful animal systems of non-alternative nature for HIV-1 study do not exist at all to date, although there are pre-existing animal models of some significance.

HIV-1 does not replicate in animal species except for chimpanzees and humans (Nomaguchi et al., 2008a). Animals frequently used for our experiments on virology, such as rodents and nonhuman primates, are not exceptions to this barrier. However, if we are to search for, develop and establish a fruitful animal model system for HIV-1 research, nonhuman primates are considered to be most suited, for HIV-1 is best fitted with humans and some apes. Ever since the discovery of HIV-1 (Barre-Sinoussi et al., 1983), many prominent researchers keen on understanding its biology and molecular biology have done investigations extensively to elucidate the bases underlying the species-specificity unique to HIV-1. These studies have highlighted the presence of potent anti-HIV-1 factors in nonhuman cells that efficiently restrict or even abolish the replication of HIV-1 and successfully raised an epoch-making notion of the intrinsic immunity (Andrew & Strebel, 2010; Arhel & Kirchhoff, 2010; Ayinde et al., 2010; Bergamaschi & Pancino, 2010; Douglas et al., 2010; Fujita et al., 2010; Huthoff & Towers, 2008; Kirchhoff, 2010; Luban, 2007; Malim & Emerman, 2008; Nakayama & Shioda, 2010; Nomaguchi et al., 2008a, 2008b; Planelles & Barker, 2010; Sauter et al., 2010; Strebel et al., 2009; Towers, 2007). Cellular factors shoulder

this intrinsic immunity known to date are cyclophilin A (CypA) (Franke et al., 1994; Thali et al., 1994), apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G)/APOBEC3F (Sheehy et al., 2002), tripartite motif protein 5α (TRIM5α) (Stremlau et al., 2004), TRIMCyp (Nisole et al., 2004; Sayah et al., 2004), and tetherin (alternatively called BST-2) (Neil et al., 2008; Van Damme et al., 2008). Because HIV-1 can indeed counteract human orthologs of these restriction factors effectively, it is well anticipated that HIV-1 in turn can be genetically engineered to replicate efficiently in nonhuman primates such as macaques. Nonetheless, most likely due to the lack of extensive and appropriate biological studies, we are still forced to use macaque-derived simian immunodeficiency virus (SIVmac) or SIVmac chimeric with a small portion of HIV-1 (SHIV) as an input virus for in vivo model studies in macaques. SIVmac and SHIV are genetically and biologically distinct from HIV-1 in a number of critical points, albeit they are quite similar to HIV-1 in the genome organization and pathogenic potentials (Desrosiers, 2007; Freed & Martin, 2007). They might not be used for future model studies aimed at understanding the biology of HIV-1 as a highly replicable/mutable, persistent, and pathogenic virus. We must go behind the outward form to grasp the inner meaning of the phenomenon, i.e., the species-specificity.

On the collective basis of molecular and biochemical studies performed by us and others so far, we recently have constructed a series of HIV-1 derivative clones tropic for macaque cells and/or macaques (Hatcho et al., 2008; Igarashi et al., 2007; Kamada et al., 2006, 2009; Kuroishi et al., 2009; Nomaguchi et al., 2008a; Saito et al., 2011; Yamashita et al., 2008), and are currently further modifying them for in vivo studies (our unpublished results). The viruses we have generated carry a minimal sequence of SIVmac, and overcome at least some species barriers. Importantly, these viruses are regarded to be genetically HIV-1, since they have less than 10% SIVmac genetic content (Igarashi et al., 2007). While we firmly believe that HIV-1 derivative viruses already constructed in our laboratory are useful for a variety of studies on HIV-1 infection in individuals, further improvement of the viruses by deliberating the evolutional process of SIV/HIV would surely add more scientific significance to basic and applied research fields. Needless to say, our goal is to generate a macaque-tropic HIV-1 (HIV-1mt) that replicates efficiently and is pathogenic for macaques as a standard pathogenic SIVmac clone such as SIVmac239 (Kestler et al., 1990). Through construction and biochemical/biological characterization of the ideal HIV-1mt clone with ability to induce AIDS at least in some species of macaques, we would be able to clarify the detailed molecular mechanisms for the narrow host range (species-tropism) of HIV-1. Viral Gag-capsid (CA) and accessory proteins (Vif, Vpx, Vpr, Vpu and Nef) are targets for those studies as a matter of course. Moreover, by using this persistent and pathogenic HIV-1mt clone as a seed virus for macaque infection experiments, we can trace and analyze its mutation, adaptation, evolutional direction to generate viral quasi-species, and finally pathogenesis in the context of immunological interaction. In addition, we can evaluate and develop the anti-HIV-1 drugs/vaccines by this HIV-1mt/macaque system.

In this chapter, we first outline the early and current studies on HIV-1, SIVmac and SHIV to emphasize and address the unique characteristics of HIV-1 and scientific issues to resolve. We then describe viral and cellular factors that are responsible for or potentially associated with restriction of viral replication. We finally focus on our recent studies on the strategies to obtain HIV-1mt clones and on the biology/molecular biology of HIV-1mt clones. Main parts of this chapter consist of: (i) Overview of the biology and molecular biology of HIV-1, SIVmac and SHIV; (ii) Determinants for HIV-1 species-tropism; (iii) Generation and

characterization of various HIV-1mt clones. The primary mission of we basic virologists is to understand viral replication and viral pathogenesis in vivo by multilateral approaches (Nomaguchi & Adachi, 2010). We then take over our new important findings to functional parties in related fields, thus promoting further progress in virology.

2. Overview of the biology and molecular biology of HIV-1, SIVmac and SHIV

Numerous immunodeficiency viruses of distinct groups have been isolated from humans and a wide variety of African nonhuman primates (Desrosiers, 2007; Freed & Martin, 2007). These viruses infect the immune system of primates, kill cells that are critical for effective immune responses, and eventually cause AIDS in some hosts (Desrosiers, 2007; Kuritzkes & Walker, 2007). Soon after the discovery in 1983 (Barre-Sinoussi et al., 1983; Barre-Sinoussi, 2010; Montagnier, 2010), HIV-1 was demonstrated to belong to a lentiviral genus of the retrovirus family, and expected to exhibit the properties characteristic of the family (Goff, 2007). In 1986, another human immunodeficiency virus was identified and designated HIV-2 (Clavel et al., 1986; Montagnier, 2010). Among these primate lentiviruses, HIV-1, HIV-2 and its close relative SIVmac are most well-studied through biological, biochemical and medical approaches, and many findings crucial for the biological and medical sciences have been generated (Ho & Bieniasz, 2008).

Basically, HIV/SIV exhibit a virological phenotype common to the retroviruses. Viral proteins are synthesized from viral DNA genome integrated into host chromosomal DNA, and progeny viral particles (virions) are produced from cells in a typical manner. However, HIV/SIV are unique, as primate lentiviruses, in the genome and virion composition among the retroviruses (Fig. 1). They all have additional genes relative to a standard retrovirus. Importantly, these extra genes encode, in addition to structural Gag, Pol and Env proteins common to all retroviruses, viral regulatory (Tat and Rev) and accessory proteins that are essential for the specific and unique characteristics of HIV/SIV. HIV/SIV virions, therefore, contain some viral proteins not found in the other retroviral virions. The common and unique properties are also applicable to their replication cycle. HIV/SIV replicate in their target cells essentially in the same way with the other retroviruses. Retroviral replication cycle consists of early and late phases. The early phase (Fig. 2) begins with the virion entry step into cells, and proceeds to the reverse transcription of viral RNA genome, uncoating, nuclear import of viral DNA genome, and integration of viral DNA genome into host DNA to generate proviral DNA. The late phase (Fig. 3) then starts with the proviral transcription, and proceeds to the viral RNA export to cytoplasm, translation into viral proteins, assembly of the viral RNA/proteins at cell surface, budding/release from cells, and maturation into infectious virions.

Of viral proteins unique to HIV/SIV, Tat and Rev are essential for virus replication as is the case for structural proteins Gag, Pol, and Env, and act as regulators for expression of the other viral proteins (Freed & Martin, 2007). Tat is a potent trans-activator of transcription, and is the primary switch of viral gene expression. Rev is responsible for the viral RNA export process, and required for expression of viral structural proteins and most of the accessory proteins except for Nef. Thus, Rev can be considered to be the second expression switch. In contrast to the two regulatory proteins, accessory proteins are not always necessary for viral replication in cells (Freed & Martin, 2007). Early studies indicated that these proteins are unnecessary or dispensable for virus replication in the established cell lines. However, it was soon noticed that, in the primary natural target cells such as CD4-positive T-lymphocytes and macrophages, or in some specific cell lines, the accessory

proteins are essential or important for virus replication. These findings have led to the identification of innate anti-viral factors APOBEC proteins (Sheehy et al., 2002) and tetherin as described above (Neil et al., 2008; Van Damme et al., 2008), and to the search for an anti-viral macrophage factor(s) (Fujita et al., 2010). Although some aspects of the accessory proteins are becoming more organized and much clearer than before as summarized in Table 1, detailed mechanisms for their activity remain to be elucidated. In particular, much is still unknown about structurally related Vpr and Vpx proteins. Moreover, functional studies in animals on HIV-1 and HIV-2 accessory proteins have not yet been performed.

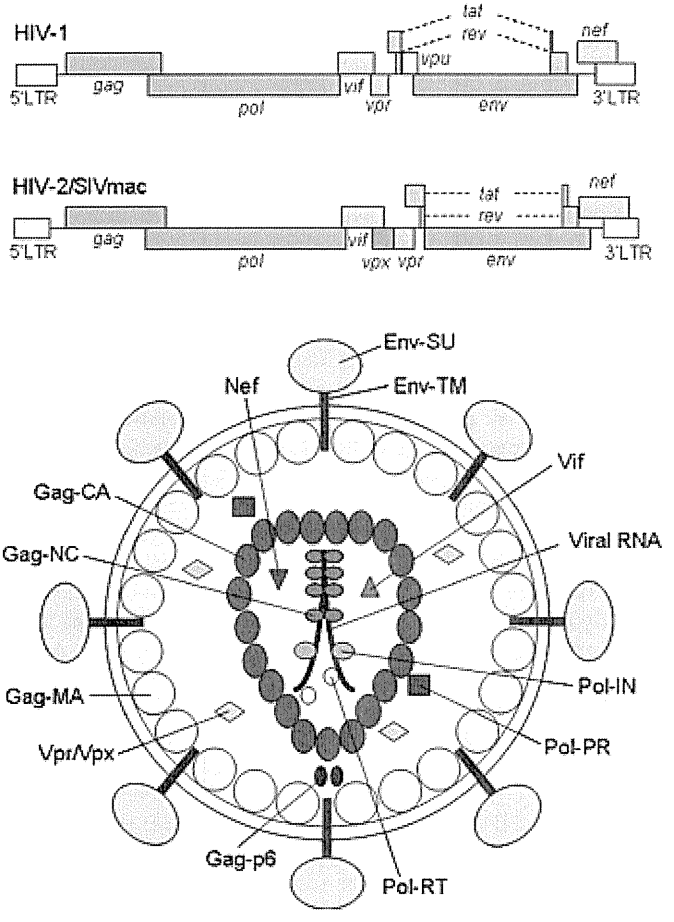

Fig. 1. Genome and virion characteristics of HIV/SIV. Upper: Proviral genome structure is schematically shown. Blue, orange, pink areas (boxes) indicate the structural, regulatory and accessory genes, respectively. Accessory genes unique to HIV-1 (*vpu*) and HIV-2/SIVmac (*vpx*) are indicated by yellow and purple, respectively. LTR, long terminal repeat. Lower: A schema of viral particle (virion). Viral proteins reported to be present in virion are illustrated. CA, capsid; IN, integrase; MA, matrix; NC, nucleocapsid; PR, protease; RT, reverse transcriptase; SU, surface; TM, transmembrane.

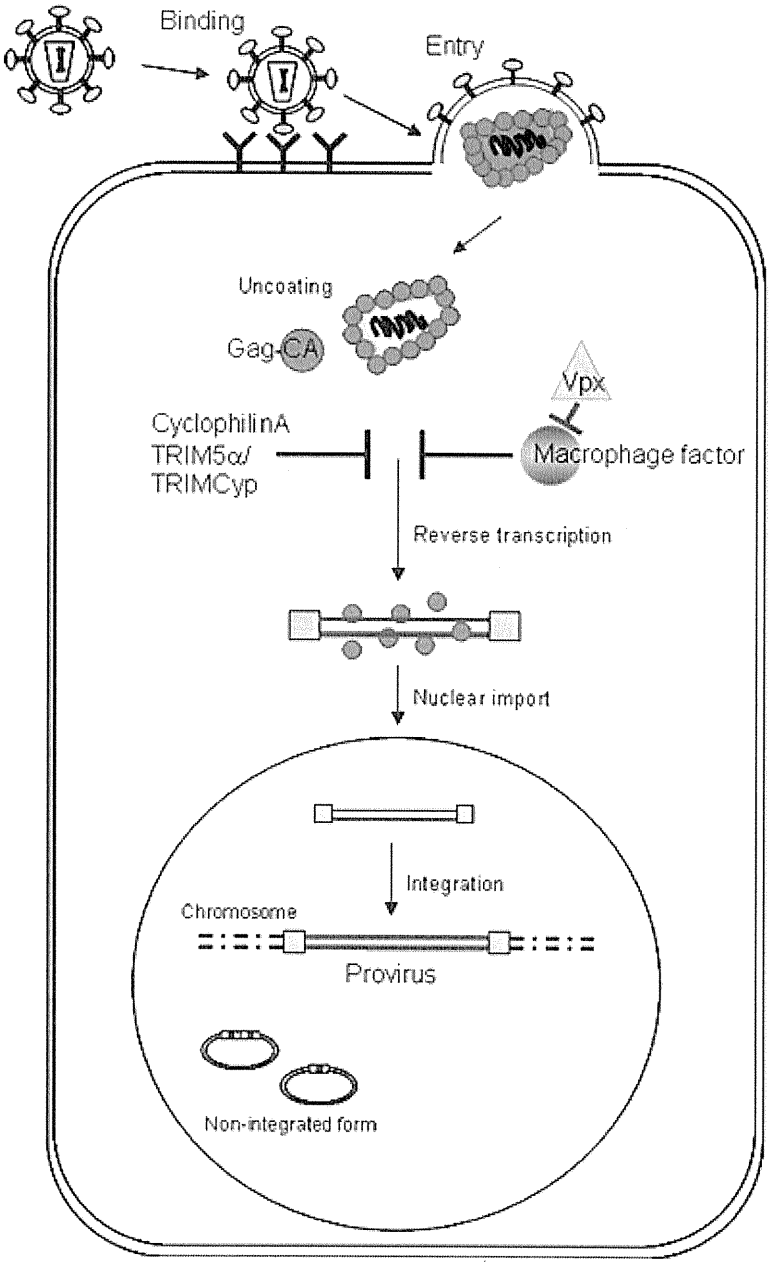

Fig. 2. The early phase of HIV/SIV replication cycle in target cells. Viral replication steps from the binding to generation of provirus are shown. Viral and cellular proteins particularly important in this chapter are highlighted. For details, see the reference (Freed & Martin, 2007).

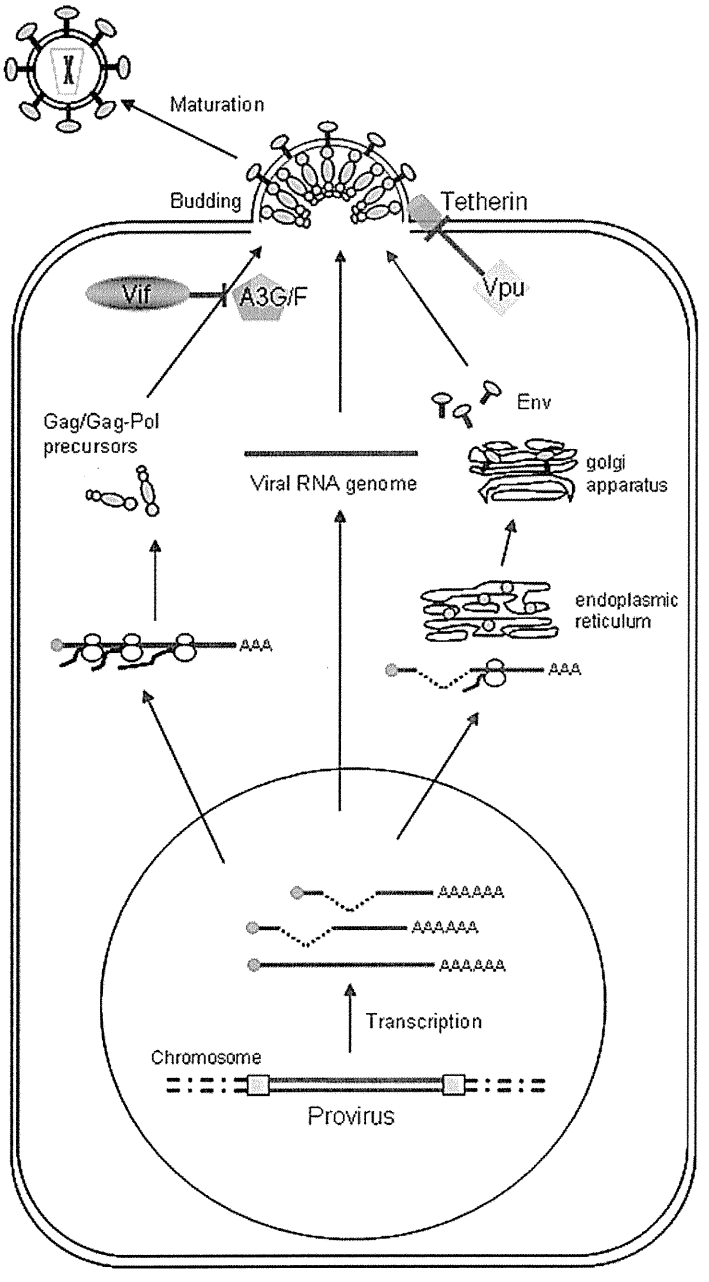

Fig. 3. The late phase of HIV/SIV replication cycle in target cells. Viral replication steps from the transcription of proviral genome to maturation are shown. Viral and cellular proteins particularly important in this chapter are highlighted. For details, see the reference (Freed & Martin, 2007).

Accessory proteins	Activities	References
Vif	Neutralization of antiviral activities of APOBEC3G/F Induction of G2 cell cycle arrest	Holmes et al., 2007 Huthoff & Towers, 2008 Izumi et al., 2010
Vpx	Inactivation of macrophage factor	Fujita et al., 2010
Vpr	Induction of G2 cell cycle arrest Trans-activation of transcription Promotion of nuclear import of pre-integration complex	Le rouzic & Benichou, 2005 Andersen et al., 2008 Ayinde et al., 2010 Fujita et al., 2010
Vpu	Degradation of tetherin Degradation of newly synthesized CD4 in ER	Bour & Strebel, 2003 Nomaguchi et al., 2008b Tokarev et al., 2009
Nef	Down-regulation of cell surface molecules (CD4, MHC-I etc.) Enhancement of viral infectivity	Kirchhoff et al., 2008 Kirchhoff, 2009 Jere et al., 2009

Table 1. Multi-functional activity of HIV/SIVmac accessory proteins. Major functions or activities are listed. For details, refer to the articles shown. ER, endoplasmic reticulum; MHC, major histocompatibility complex.

One of the most outstanding biological properties of HIV-1 is its especially narrow host range. It was recognized soon after the virus isolation that HIV-1 can not infect macaque cells and macaques, animals frequently used for experimental infection. We, therefore, pioneered the work to determine viral determinants for this species-tropism by construction and characterization of chimeric viruses between SIVmac and HIV-1 (Nomaguchi et al., 2008; Sakuragi et al., 1992; Shibata et al., 1991, 1995; Shibata & Adachi, 1992). SIVmac has a wider host range relative to HIV-1, and can efficiently replicate both in macaque and human cells. The chimeric viruses (Fig. 4), later called SHIV, were useful to localize the viral genetic area responsible for the tropism. Among NM-1, NM-3, and NM-8 in Fig.4, only NM-3 was shown to display infectivity to macaque cells. In addition, Gag-CA region was suggested to be important for the tropism by a similar analysis of chimeric viruses (Dorfman & Gottlinger, 1996). Totally, these SHIV studies revealed that Gag-CA plus some viral protein(s) encoded by the central viral genomic region may determine the HIV-1 species-tropism.

As for input viruses of model infection studies in macaques, SIVmac and SHIVs have been widely and frequently used (Ambrose et al., 2007; Nomaguchi et al., 2008). SIVmac is thought to emerge by a cross-species infection of rhesus macaques with SIVsmm naturally occurring in African sooty mangabeys (Fultz et al., 1986; Murphey-Corb et al., 1986). It targets CD4-positive cells such as T-lymphocytes and macrophages, persists, and finally cause AIDS in rhesus macaques. Pathogenic SHIVs have been obtained from the original prototype SHIV by serial animal passages, and were used for infection experiments in rhesus macaques. An SIVmac derivative that has reverse transcriptase (RT) of HIV-1 (RT-SHIV) (Fig. 4) was also constructed to test the effect of anti-RT drugs on virus replication (Uberla et al., 1995). Although these viruses did contribute much to HIV-1 model studies

including the assessment of immune response, evaluation of anti-viral drugs, analysis of drug-resistance, and establishing the strategy for vaccine development, there are some intrinsic differences among important virological properties of HIV-1, SIVmac and SHIVs as summalized in Table 2. These should be seriously considered for the future model studies. To underscore the essential need for the suitable primate model research to answer basic questions about HIV-1 in vivo, we wish to mention here, as an example, that the trials to develop anti-viral vaccines have been unsuccessful due to the lack of appropriate models (Hayden, 2008; Watkins et al., 2008).

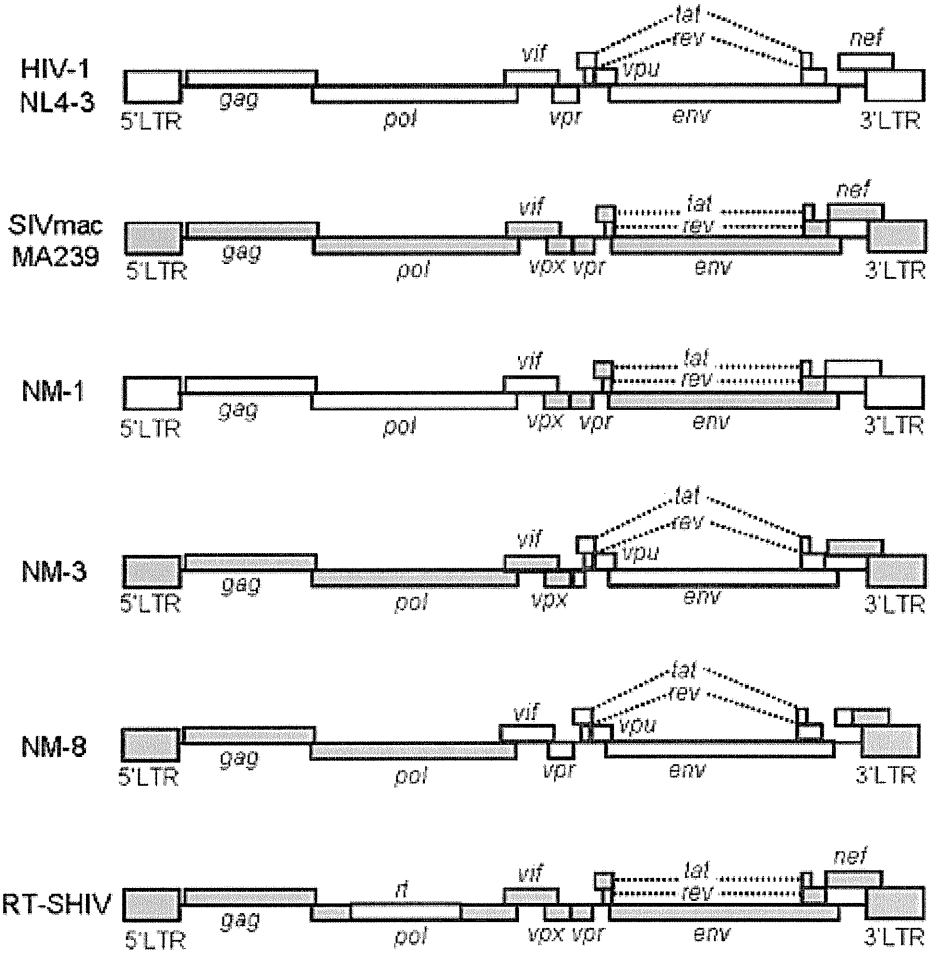

Fig. 4. Genome organization of HIV/SIV and representative SHIVs. Proviral genome structure is schematically shown. White and blue areas (boxes) indicate the genes and LTR of HIV-1 NL4-3 (Adachi et al., 1986) and SIVmac MA239 (Shibata et al., 1991), respectively. Areas without gene names indicate that the genes there are inactivated by genetic manipulations.

	HIV-1/human	SIVmac/rhesus	SHIV/rhesus	References
Outcomes in individuals				
Response to vaccines			Easily vaccinated	Ambrose et al., 2007 Freed & Martin, 2007
Response to drugs		Not always inhibited by anti-HIV-1 drugs	Not always inhibited by anti-HIV-1 drugs	Ambrose et al., 2007 Uberla et al., 1995
Median disease course	Approximately 10 years	One to three years	Rapid	Brown et al., 2007 Freed & Martin, 2007
Emergence of X4-tropic isolates	Frequent (Subtype B)	Rare		Brown et al., 2007
Accessory proteins in cells				
Vpx	Not present	Inactivate an unidentified anti-viral factor in macrophages		Fujita et al., 2008 Sharova et al., 2008
Vpr	Influence viral replication in macrophages	Act on viral replication nothing in macrophages		Fujita et al., 2010
Vpu	Antagonize tetherin	Not present		Neil et al., 2008
Nef	Not antagonize tetherin Not down-regulate CD3	Antagonize tetherin Down-regulate CD3		Zhang et al., 2009 Kirchhoff, 2009

Table 2. Biology of HIV-1, SIVmac, and SHIV in infected hosts. For details, see the main references shown.