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Original article

Construction of a novel SHIV having an HIV-1-derived protease gene and its infection to rhesus macaques: a useful tool for in vivo efficacy tests of protease inhibitors

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Abstract

We generated a novel SHIV (termed SHIV-pr) that possesses the HIV-1-derived protease (PR) gene in the corresponding position in the SIVmac genome. SHIV-pr is replication-competent in human and monkey $CD4^+$ T lymphoid cell lines as well as rhesus macaque PBMCs. The viral growth of SHIV-pr was completely blocked in the presence of a peptide-analog PR inhibitor at the tissue culture level. When SHIV-pr was intravenously inoculated into two rhesus macaques, it resulted in a weak but long-lasting persistent infection in one monkey, whereas the infection of another was only temporary. To enhance the viral growth competence by adaptation, we then passaged the virus in vivo from a monkey up to the fourth generation. The initial peak values of plasma viral loads as well as the setpoint values increased generation by generation and reached those of a parental virus SIVmac. When a medication using the content of Kaletra capsule (a mixture of two PR inhibitors, lopinavir and ritonavir) was orally given to three SHIV-pr-infected monkeys for 4 weeks, plasma viral loads dropped to near or below the detection limit and quickly rebounded after the cessation of medication. The results suggest that SHIV-pr can be used to evaluate PR inhibitors using monkeys.

Keywords: SHIV; Chimeric virus; Protease; Inhibitor; Animal model; Monkey

1. Introduction

Since a new therapy called highly active anti-retroviral therapy (HAART) started to cure acquired immunodeficiency syndrome (AIDS) in the latter half of the 1990s, a more or less optimistic perspective that AIDS has become a controllable sickness has spread among people. HAART has truly exhibited its dramatic effect to suppress the disease progression and prolong the lives of the patients; but now it is confronting numerous difficulties such as how to suppress emergence of multi-drug resistant viruses and how to avoid the severe side effects caused by long-term uptake of a large amount of drugs every day [1]. Thus, there is an urgent need to test new therapeutics.

One approach is to develop drugs against the human immunodeficiency virus type 1 (HIV-1)-encoded protease (PR) gene, which is essential for viral replication. Such drugs can be tested in human tissue cultures, but the results do not reliably predict how the drugs will work in vivo. An animal model would be the best means for testing such drugs. Simian immunodeficiency virus (SIV) has been widely used with macaque monkeys since macaques are rather easily available for researchers and since the virus, SIVmac, causes AIDS-like

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symptoms within 2–3 years post-infection [2]. However, a problem with the SIVmac/monkey model in testing PR inhibitors is that the sensitivity of SIVmac PR to HIV-1-targeted PR inhibitors may not be as good as that of HIV-1 PR probably because of structural differences of the respective PRs. Thus we constructed a novel SIV/HIV chimeric virus (SHIV), in which only the PR gene is derived from HIV-1.

Here, we show that the new virus, SHIV-pr, was able to replicate in monkeys, indicating that the HIV-1 PR could function with SIV, and that oral administration of Kaletra, a mixture of two HIV-1 PR inhibitors (lopinavir and ritonavir), reduced plasma viral loads to near or below the limitation of detection. These results indicate that SHIV-pr can be used to test the in vivo efficacy of anti-HIV-1 PR inhibitors in monkeys.

2. Materials and methods

2.1. DNA constructs

Infectious molecular clones of HIV-1 (pNL432) [3] and SIVmac239 (pMA239) [4] were used as parent proviral DNAs in this study. The genomic organization of SHIV-pr, in which the PR gene of SIVmac was replaced by that of HIV-1, is shown in Fig. 1A, and its more detailed structure is shown in Fig. 1B. Chimeric junctions were generated at the N-terminal and C-terminal ends of the viral PR gene by polymerase chain reaction (PCR)-based site-directed mutagenesis as follows. First, the *Spe I* (nt 1507)-*Sse 8387I* (nt 2839) gene fragment of pNL432 and the *Spe I* (nt 2026)-*Sse 8387I* (nt 3397) gene fragment of pMA239, both of which span the region from the middle of the capsid protein (CA) gene to the N-terminal region of reverse transcriptase (RT) gene, were cut out by restriction enzymes and then inserted into pUCTA119 [5], which was modified from pUC119 to possess an *Spe I* site at the multiple cloning site and precut by *Spe I* and *Pst I*. The viral gene-inserted plasmids were named pHIV-CAPR and pSIV-CAPR, respectively. Then PCR was run using a primer SHPR-F 5′-TTA*GTGCAC*CTCAGAT CACTCTTTGGCAGCGACCCC-3′ and an M13 forward primer and pHIV-CAPR as a template. Digestion of this PCR product with *Bsp 1286I* (*Bsp 1286I* site italicized) and *Sse 8387I* yielded a fragment containing the HIV-1 PR genomic region with a sticky ACGT overhang at the N-terminal side of the PR gene. Another PCR was run using a primer SHPR-R 5′-TTGAG*CTGCAG*CAAATCCTCTGTCACCTCC-3′ and an M13 reverse primer and pSIV-CAPR as a template. Digestion of this PCR product with *Spe I* and *Pst I* (*Pst I* site italicized) yielded a fragment containing the 3′ half of the SIVmac *gag* gene with a sticky TGCA overhang near the C-terminal side of the *gag* gene. The two fragments obtained were ligated and subcloned into pUCTA119 precut with *Spe I* and *Pst I*. By doing so, a SIVmac/HIV-1 chimeric junction was made at the N-terminus of the PR gene. To generate a chimeric junction at the C-terminus of the PR gene, we first introduced a *Dra I* site near the C-terminus of the PR gene of pSIV-CAPR because pNL432 (HIV-1) already possesses the same site exactly at the corresponding position (nt 2540) and creating

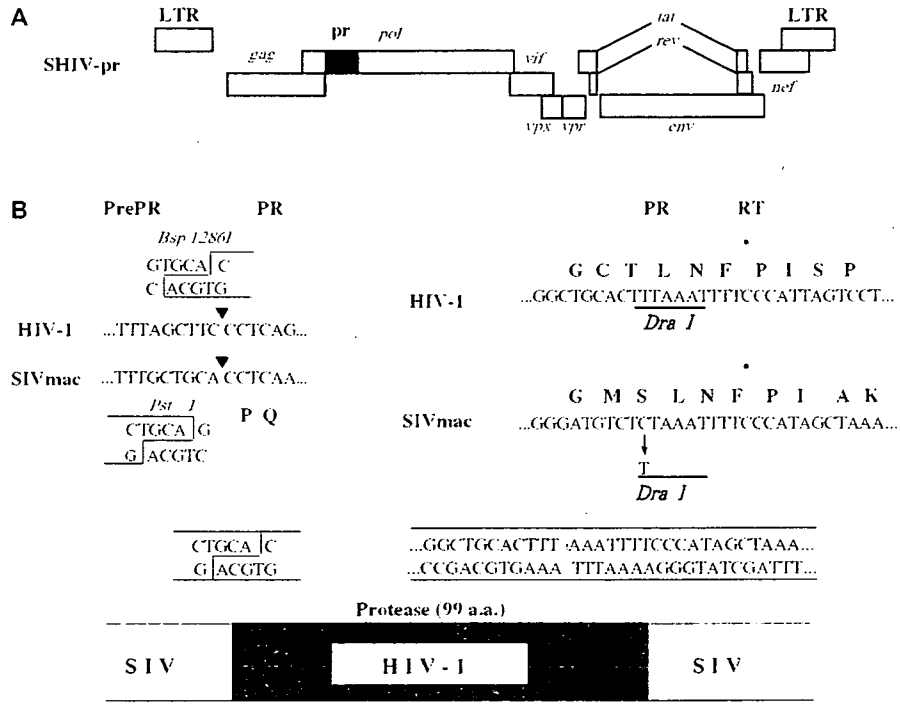

Fig. 1. Construction of SHIV-pr having the HIV-1 PR gene in the SIVmac genome. (A) Genomic organization of SHIV-pr. The HIV-1-derived genomic region is indicated by a black bar. (B) Nucleotide sequences near the N-terminal and C-terminal ends of PR genes of HIV-1 and SIVmac. The arrows indicate the cleavage sites by viral protease. The detailed procedures of construction of chimeric junctions in SHIV-pr are described in Section 2.

a *Dra I* made it easy to produce the chimeric junction. This mutation itself does not alter the amino acid sequence of PR. PCR was run using a primer MAPR-R 5′-CTTTAGCTA TGGGAAAA*TTTAAA*GACATCCCCAGAGCTG-3′ (*Dra I* site italicized) and an M13 reverse primer and pSIV-CAPR as a template. After creating a *Dra I* site, an HIV-1/SIVmac chimeric junction at the C-terminus of the PR region was made by conventional molecular techniques. Finally, the *Spe I-Sse 8387I* fragment having the HIV-1 PR gene in a SIVmac background was transferred to the corresponding position of pMA239. This molecular clone of a novel SHIV having the HIV-1 PR gene in the SIVmac genome was termed pSHIV-pr. A *Dra I*-incorporated mutant full-genome plasmid of pMA239 was also made and named pSIVmac-mD.

2.2. Cell cultures

M8166 is a subclone of C8166 [6], a $CD4^+$ human T-cell line. HSC-F is a cynomolgous monkey $CD4^+$ T-cell line from a fetal splenocyte that was immortalized by infection with *Herpesvirus saimiri* subtype C [7]. M8166 cells and HSC-F cells were maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS). PBMCs of healthy rhesus monkeys were separated from heparinized whole blood by Percoll density gradient centrifugation, stimulated with 25 mg/ml of concanavalin A for 24 h, and maintained in RPMI 1640 medium containing 10% FBS and 400 U/ml of recombinant human interleukin 2 (IL-2) as described previously [8].

2.3. Transfection and infection

To generate infectious virus particles from a full-genome plasmid DNA, typically 5 μg of pSHIV-pr was introduced into 1.5 10^6 M8166 cells by the DEAE-dextran method [9]. The culture supernatants were harvested every 3 days and stored at 80 C. Then, virion-associated RT activity was measured as described previously [10]. The fractions with the highest RT activities were pooled, filtered (0.45 μm pore size) and stored as a virus stock at 80 C. To determine the viral infectivity of the virus stock, the 50% tissue culture infectious dose ($TCID_{50}$) was calculated by using M8166 cells. The virus inoculum used for in vitro infection was adjusted to contain 3–4 10^3 RT units by adding the appropriate volume of the medium to the virus stock. M8166 cells, HSC-F cells or monkey PBMCs (5 10^4 cells/well) were infected with a virus and cultured in a 96-well plate. The culture supernatants were harvested every 3 days and production of the virions was monitored by measuring their RT activities.

2.4. Western blotting

Expression of the viral proteins after transfection by the DEAE-dextran method was examined by Western immunoblotting. M8166 cells were transfected with proviral plasmids and the cell lysates were prepared, resolved on 12% polyacrylamide gels and Gag precursor polyproteins and its protease-cleaved products were detected by Western immunoblotting using a SHIV-3rN-inoculated antiserum [8]. SHIV-3rN-inoculated monkeys developed strong high titers of antibodies against the viral component proteins especially Gag and Pol proteins. Antiserum of these monkeys was used to detect the SIVmac-derived viral structural proteins.

2.5. KNI-272, a PR inhibitor

KNI-272, a peptide-analog PR inhibitor [11], was kindly provided by Prof. Yoshiaki Kiso, Department of Medical Chemistry, Kyoto Pharmaceutical College, Kyoto, Japan.

2.6. Inoculation of macaques and in vivo passage

Six rhesus macaques (*Macaca mulatta*) negative for SIV and simian T-cell lymphotropic virus were used in the present study. All the monkeys were intravenously inoculated with SHIV-pr through the foot saphenous vein. First we inoculated a cell-free virus stock of SHIV-pr (2 10^5 $TCID_{50}$) to two rhesus macaques (MM236 and MM239 [first]). For the second generation, we injected plasma taken from MM239 at 2, 27, and 51 weeks post-infection (w.p.i.) into MM275 (a flowchart of in vivo passages is given in Fig. 4C). For the third generation we injected plasma and minced lymph nodes from MM275 at 17 days post-infection into MM274. For the fourth generation, we injected a mixture of plasma and isolated virus from PBMCs of MM274 at 3 w.p.i. into MM289 and MM294. All animals were housed in a P3-level monkey storage facility, and were treated in accordance with regulations approved by the Committee for Experimental Use of Nonhuman Primates in the Institute for Virus Research, Kyoto University.

2.7. Flow cytometry

The $CD4^+$ and $CD8^+$ T lymphocyte subset in PBMCs were examined by flow cytometry as previously described [12].

2.8. Virus isolation

We attempted to isolate infectious viruses from the PBMCs of inoculated monkeys as follows. PBMCs (typically 1 10^6 cells) were cocultured with 1 10^6 M8166 cells for at least 4 weeks in RPMI 1640 containing 10% of FBS in a 24-well plate. Virus recovery was judged by the syncytial cytopathic effect (CPE) by a microscopic observation and a rise of RT activity of the culture supernatants.

2.9. Determination of plasma viral RNA loads

Plasma viral RNA loads were determined by quantitative RT-PCR as described previously [12]. The detection limit was 500 copies/ml.

2.10. Titration of antibody

Antibody titers of the monkey sera after the inoculation with SHIV-pr were measured by particle agglutination

according to the instructions of the manufacturer (Serodia HIV-1/2, Fujirebio, Tokyo, Japan).

2.11. Protease inhibitor treatment in vivo

The contents of a Kaletra capsule (a mixture of lopinavir and ritonavir, Abbot, Japan) was suspended in drinking water and administered by the oral route (12 mg of lopinavir and 3 mg of ritonavir/kg/day) to three monkeys (MM275, MM289 and MM294) for 4 weeks.

3. Results

3.1. Autoprocessing of Gag proteins of SHIV-pr

To clarify whether the HIV-1-derived PR could undergo authentic proteolytic processing of Gag precursor polyproteins, we examined lysates of virus-infected cells with Western blot. As shown in Fig. 2, autoproccessing patterns of p55 Gag precursor proteins of SHIV-pr, SIVmac, and SIVmac-mD were basically equal and bands of p26 capsid and p17 matrix proteins as well as p41 partially processed intermediate products were clearly detected, suggesting that the PR of SHIV-pr can recognize and cut the cleavage sites although its proteolytic activity seems to be slightly lower as judged by the band intensities.

3.2. Replication of SHIV-pr at the in vitro tissue culture level

The growth-competences of SHIV-pr as well as its parental viruses HIV-1 and SIVmac were examined in human and monkey lymphoid cell lines, M8166 and HSC-F, respectively (Fig. 3). As shown in Fig. 3A, SHIV-pr replicated fairly well, with showing conspicuous ballooning in M8166 cells although the start and peak of viral growth were delayed by several days compared with those of SIVmac. SHIV-pr was also replication-competent in a cynomolgous monkey-derived cell line, HSC-F cells; whereas HIV-1 could not replicate at all (Fig. 3B). In this monkey cell line, the growth of SHIV-pr was obviously delayed compared with that of SIVmac, but more importantly SHIV-pr exhibited an ability to replicate in monkey cells.

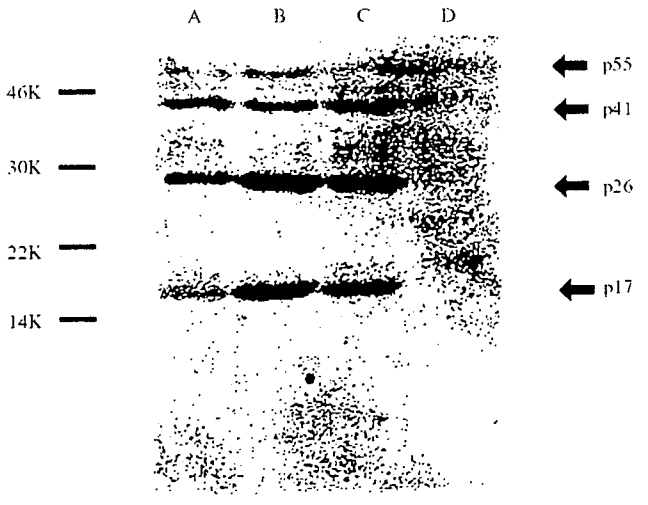

Fig. 2. Immunoblotting analysis of proteolytic processing of Gag proteins. The lysates of M8166 cells transfected with plasmids of a chimeric and a parental viruses were harvested at 48 h and resolved on a 12% SDS-polyacrylamide gel. Immunoblotting was done by use of a plasma of a monkey infected with a chimeric virus NM-3rN [8]. Lanes A–D represent the lysates of SHIV-pr (A), SIVmac (B), SIVmac-m D (*Dra I*-incorporated mutant) (C), and mock infection (D).

3.3. Effect of a PR inhibitor, KNI-272, on the viral growth of SHIV-pr and SIVmac in HSC-F cells and macaque PBMCs

To clarify whether a PR inhibitor, KNI-272, is effective on the growth of SHIV-pr and its parental SIVmac or not, the growth-kinetics in HSC-F cells and lower macaque PBMCs were examined in the presence and absence of the inhibitor. KNI-272 is a tripeptide-like protease inhibitor which mimics the amino acid sequence of the cleavage site between p17 matrix and p24 capsid proteins in Gag precursor proteins. The replication of SIVmac was slightly inhibited by KNI-272 at a concentration of 100 nM and blocked at a concentration of 1000 nM in HSC-F cells (Fig. 3C). On the other hand, the growth of SHIV-pr was completely inhibited by the inhibitor even at a concentration of 100 nM in both HSC-F cells and macaque PBMCs (Fig. 3C,D, respectively).

3.4. Infection of SHIV-pr to macaque monkeys

Since SHIV-pr was found to be replication-competent in monkey PBMCs, we then inoculated the virus into two macaques (MM236 and MM239). The results are summarized in Table 1. The virus was reisolated from both monkeys. MM239 showed strong induction of antibodies judged by PA tests while MM236 showed only a slight rise of PA titer. The plasma viral RNA loads of both monkeys were within a range of 10^3–10^4 copies/ml from 2 to 12 weeks post-infection (see Fig. 4A for the data of MM239), but thereafter decreased below the detection limit (500 copies/ml) except that the virus was reisolated from MM239 alone and the RNA load was 10^4 copies/ml at 27 weeks post-infection. MM239 died at 51 weeks. However, post-mortem examination did not reveal any signs of AIDS-like symptoms except a slight trait of pneumonia in the lungs, and the CD4 cell counts remained within the normal range. Overall, the inoculation of SHIV-pr seemed to have resulted in a rather weak systemic infection.

3.5. In vivo passage

Since SHIV-pr did not productively replicate in the initially inoculated monkeys, we conducted four in vivo passages to enhance viral productivity. The results are summarized in Fig. 4 (A: plasma viral RNA loads; B: CD4 cell counts; C: a scheme of passages). As shown in Fig. 4A, SHIV-pr

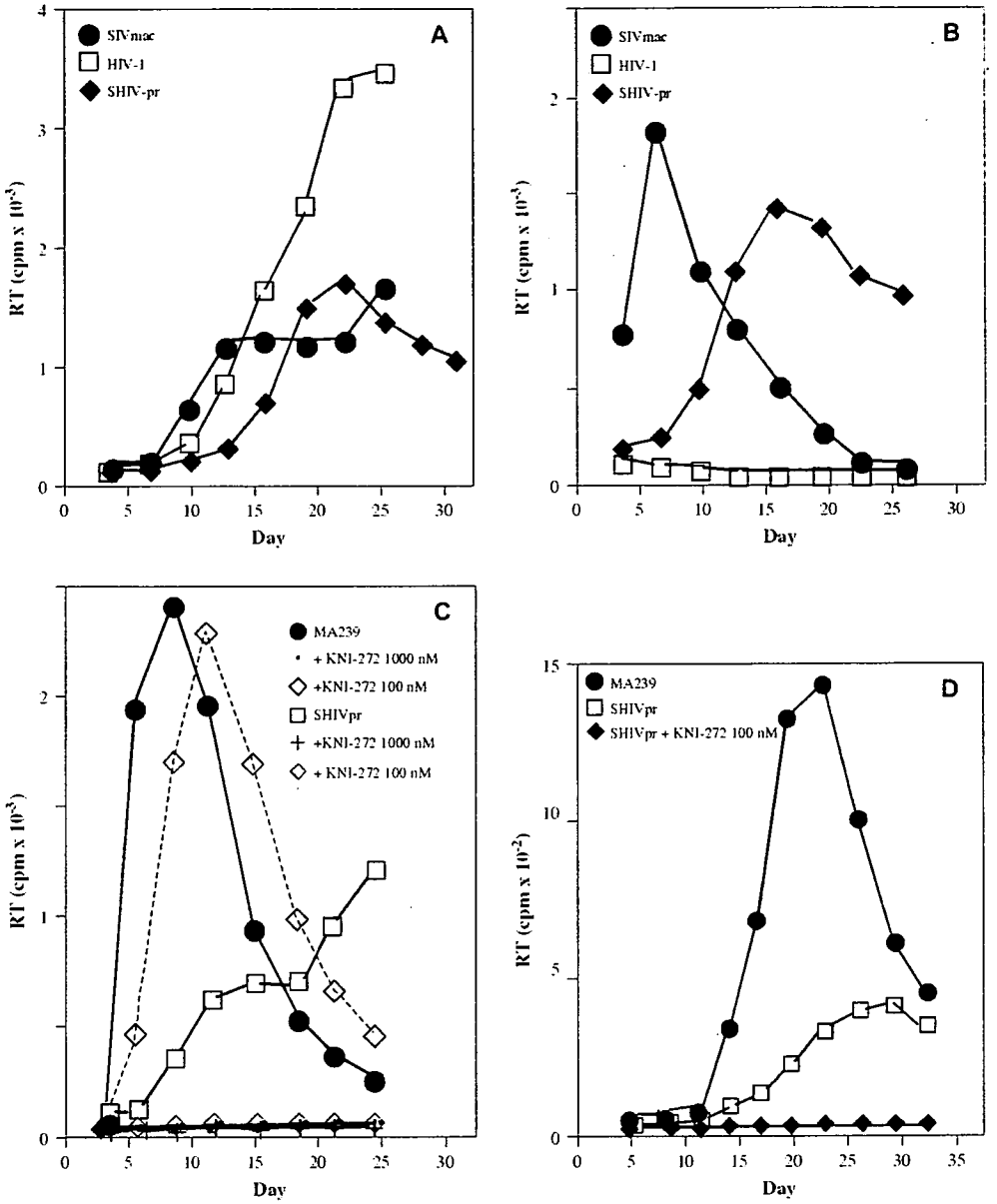

Fig. 3. Growth-kinetics of SHIV-pr and effects of a peptide-analog PR inhibitor, KNI-272. A portion of the stock virus (3–4 10^3 RT units, equivalent to approximately 1 10^4 $TCID_{50}$) of either SHIV-pr, SIVmac, or HIV-1 was inoculated on to M8166 cells, a human $CD4^+$ lymphoid cell line (A) and HSC-F cells, a cynomolgous monkey $CD4^+$ cell line (B). Viral growth was monitored by measuring the virion-associated RT activities in the culture supernatants which were periodically harvested. Mean values of three independent experiments of growth profiles are plotted. The effects of KNI-272 (0, 100 and 1000 nM) on the viral growth were also examined by measuring the RT activities in the culture media. Mean values of two independent experiments of growth profiles in HSC-F cells (C) and rhesus PBMCs (D) are plotted.

gradually enhanced its viral productivity and the peak values of viral RNA loads reached 10^5 copies/ml in the second generation. In the third (MM274) and fourth (MM289 and MM294) generations, it reached the values of 10^6 or 10^7 copies/ml with rather high setpoints (around 10^5 copies/ml) which are comparable to those of SIVmac. At 4–6 weeks post-infection, $CD4^+$ T cells of four of five monkeys decreased to approximately the 50% level but gradually recovered to the normal level (Fig. 4B).

3.6. Administration of Kaletra by the oral route to SHIV-pr-infected monkeys

Whether SHIV-pr-infected monkeys respond to protease inhibitors or not critically determines the value of this monkey model system. Now that we had monkeys with steady setpoints (MM289, MM294, and MM275), we examined the effect of the PR inhibitor mixture Kaletra on their viral loads and $CD4^+$ T cell counts.

Table 1
Virus isolation and PA antibody titers in SHIV-pr-infected rhesus monkeys (first generation)

Week	0	1	2	3	4	6	8	11	15	20	27	31	45	51
Virus isolation[a]														
MM236			+											
MM239			+	+	+	+					+			
PA titer[b]														
MM236	<32	<32	<32	32	32	32	256	256	256	256	256	256	256	256
MM239	<32	<32	<32	<32	<32	<32	256	1024	1024	1024	1024	4096	16,384	16,384

[a] PA titers were measured by a Genedia HIV-1/2 kit (Fujirebio, Inc., Japan).
[b] Virus isolation was done by co-culturing with a human T-lymphoid CD4$^+$ cell line, M8166 cells followed by reverse transcriptase activity assay.

The effect of Kaletra treatment was dramatic, causing the plasma viral RNA loads of all three monkeys to rapidly decrease to nearly the detection limit or below (Fig. 5A). However, the viral loads quickly rebounded to the levels after the cessation of drug administration. Especially in MM289, the load rebounded to the pre-treatment level within 1 week and maintained its high value thereafter. In the other two monkeys, the loads also rebounded but they did not reach the pre-treatment levels. The numbers of CD4$^+$ T cells did not change during medication in any of the monkeys. However, from 2 weeks after the cessation of medication, the number of CD4$^+$ T cells in MM294 tended to recover in MM294. No significant changes of CD4$^+$ T cell counts were observed in MM275 or MM289 (Fig. 5B).

4. Discussion

In the present study we constructed a SHIV having the PR of HIV-1 in the SIVmac genome. The generated SHIV-pr was found to be replication-competent in not only a human cell

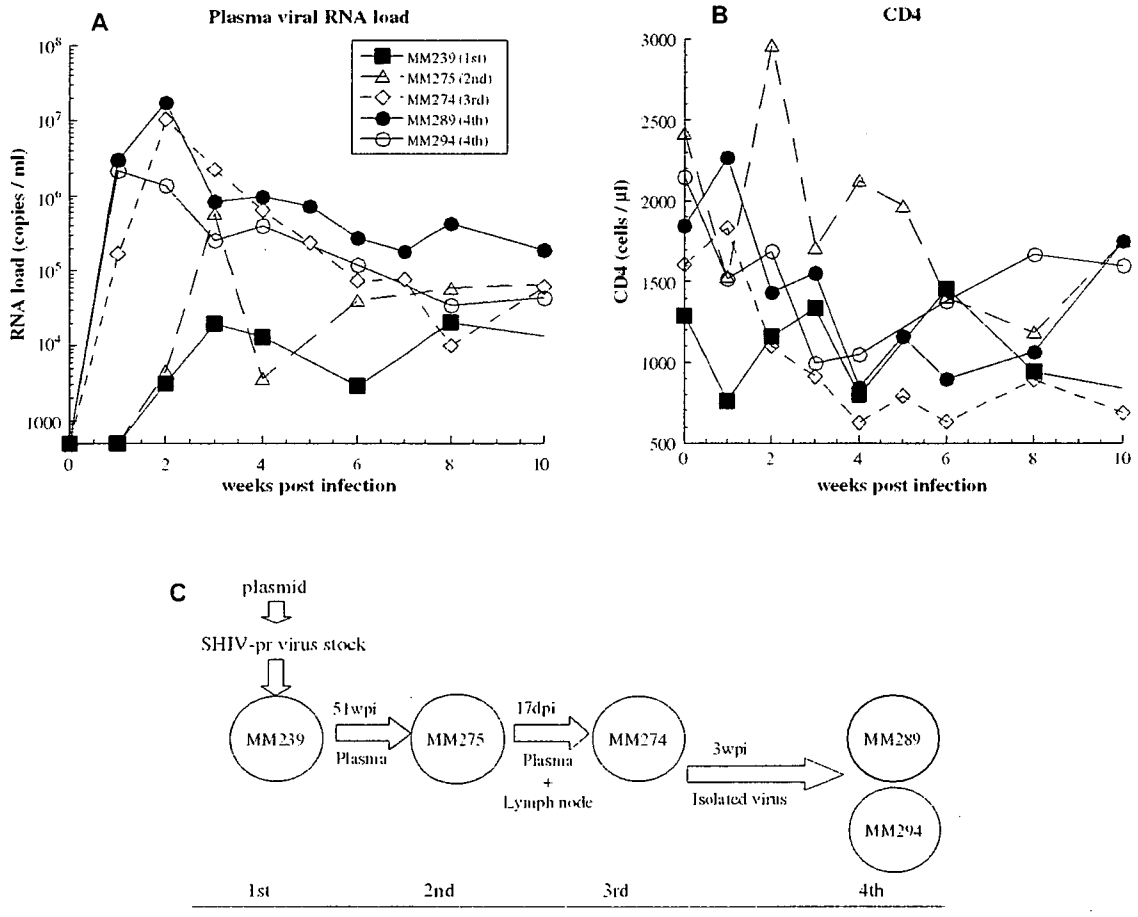

Fig. 4. Enhancement of viral productivity of SHIV-pr by in vivo passage. (A) Plasma viral RNA loads (copies/ml) of the respective generation of passages are plotted. (B) CD4$^+$ T cell counts (cells/μl) were plotted. (C) A flowchart of in vivo passages.

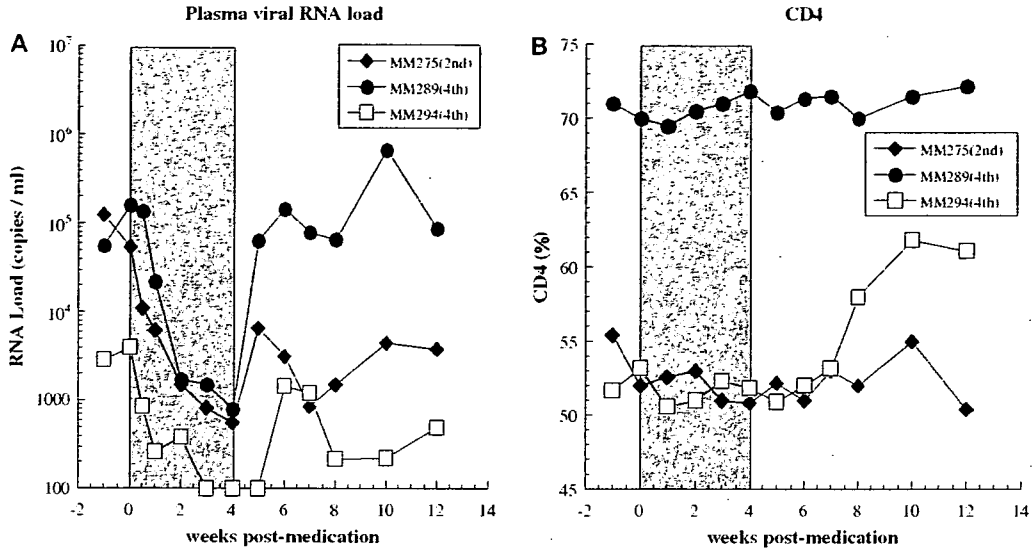

Fig. 5. Medication of SHIV-pr-infected monkeys by Kaletra (a mixture of PR inhibitors, lopinavir and ritonavir, Abbot, Japan). The contents of the Kaletra capsule were suspended in drinking water administered by the oral route (12 mg of lopinavir and 3 mg of ritonavir/kg/day) to three monkeys (MM275, MM289 and MM294) for 4 weeks (shaded). (A) Plasma viral RNA load values. (B) The ratio of $CD4^+$ T cells to $CD3^+$ T cells.

line but also a monkey cell line. The virus could also replicate even in monkeys. This is the first report of a SHIV with the PR gene of HIV-1 that is growth-competent in macaques. As revealed in Western immunoblotting, the replacement of PR did not significantly affect the autoproccessing of Gag polyproteins, suggesting that HIV-1 PR can recognize the same cleavage sites of SIVmac polyproteins as SIVmac PR. However, the growth-potential of SHIV-pr seemed to be less than that of the parental virus SIVmac based on the delay of the rise of RT activities and a lower peak value in the growth-kinetic profiles. The slightly lower activity of PR of SHIV-pr observed in Fig. 2 could partly contribute to the delayed virus growth during propagation in vitro (perhaps in vivo too) observed in Fig. 3. Further studies are needed to understand the mechanism of this weakening of viral productivity.

Inoculation of SHIV-pr to macaque monkeys resulted in a rather weak systemic infection. The replacement of PR might have disrupted the smooth maturation of viral structural proteins and consequently weakened the viral replication ability. Nonetheless, the in vivo passage clearly conferred a robust productivity to the virus. We are currently attempting to identify the mutations responsible for the increased productivity. A molecular clone of the monkey-adapted virus should also be pursued as the next step. Our main finding is that SHIV-pr could replicate in macaques and was sensitive to protease inhibitors. Our results demonstrate that the SHIV-pr/monkey system can be used for in vivo efficacy tests of protease inhibitors.

As an example of a SHIV that has chimeric boundaries in either *gag* or *pol* genomic region, RT-SHIV [13] is already known. RT-SHIV possesses the HIV-1-derived RT gene and it could replicate in macaques. The RT inhibitors are classified into two groups: nucleotide RT inhibitors (NRTIs) and non-nucleotide RT inhibitors (NNRTIs). NRTIs bind to the catalytic site and therefore they can work on both HIV-1 and SIV. On the other hand, NNRTIs bind to the RT molecules in a type-specific manner. Consequently most NNRTIs do not inhibit the replication of SIV. RT-SHIV was almost as sensitive to NNRTIs in vivo as it was in vitro [14]. In fact, the virus was used to evaluate NNRTIs [15,16]. Because SHIV-pr can be used for a similar purpose, it would be interesting to generate a new SHIV that possesses the whole *pol* genomic region of HIV-1 and to use it to conduct experiments of the HAART model.

Two groups recently developed viruses whose genomes are mostly derived from HIV-1 [17,18]. APOBEC3 family proteins and TRIM5α have recently been identified as molecules that determine species tropism. These findings may help to create viruses that differ from the HIV-1 genome by only one or two genes, such as the capsid and *vif* genes. These viruses can replicate in monkey-derived cells and possibly in monkey PBMCs. However, to the best of our knowledge, the viruses cannot replicate in rhesus macaques. Because SHIV-pr can replicate in rhesus macaques with steady and rather high values of setpoints, it appears to be best suited for evaluating HIV-1-targeted PR inhibitors in monkeys.

Acknowledgements

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A genetically engineered live-attenuated simian–human immunodeficiency virus that co-expresses the RANTES gene improves the magnitude of cellular immunity in rhesus macaques

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Abstract

Regulated-on-activation-normal-T-cell-expressed-and-secreted (RANTES), a CC-chemokine, enhances antigen-specific T helper (Th) type-1 responses against HIV-1. To evaluate the adjuvant effects of RANTES against HIV vaccine candidate in SHIV-macaque models, we genetically engineered a live-attenuated SHIV to express the RANTES gene (SHIV-RANTES) and characterized the virus's properties *in vivo*. After the vaccination, the plasma viral loads were same in the SHIV-RANTES-inoculated monkeys and the parental *nef*-deleted SHIV (SHIV-NI)-inoculated monkeys. SHIV-RANTES provided some immunity in monkeys by remarkably increasing the antigen-specific $CD4^+$ Th cell-proliferative response and by inducing an antigen-specific IFN- ELISpot response. The magnitude of the immunity in SHIV-RANTES-immunized animals, however, failed to afford greater protection against a heterologous pathogenic SHIV (SHIV-C2/1) challenge compared to control SHIV-NI-immunized animals. SHIV-RANTES immunized monkeys, elicited robust cellular $CD4^+$ Th responses and IFN- ELISpot responses after SHIV-C2/1 challenge. These findings suggest that the chemokine RANTES can augment vaccine-elicited, HIV-specific $CD4^+$ T cell responses.

Keywords: SHIV; HIV-1; SIV; RANTES; Adjuvant; Vaccine; Chemokine

Introduction

Development of an effective vaccine against HIV-1 has been a major priority to control the worldwide AIDS epidemic. To achieve this goal, several approaches are being tried. Chimeric simian and human immunodeficiency virus (SHIV) clones containing the HIV-1 *env* genes on a simian immunodeficiency virus (SIV) are useful models for HIV-1 vaccine development, because SHIVs are readily infectious to macaque monkeys, and show induction of immune responses to HIV-1 Env. We previously reported the *in vivo* properties of SHIV-NM3rN (derived from HIV-1 NL432 and SIV mac239) with deletion in the *vpx*, *vpr*, and/or *nef* genes (Igarashi et al., 1998). These gene-deleted SHIVs are candidates for vaccines against HIV-1 because attenuated SHIVs can induce anti-HIV-1 humoral and cell-mediated immunity in monkeys without AIDS-like diseases (Haga et al., 1998; Kuwata et al., 2001). Moreover, the monkeys immunized with the *nef*-deleted SHIVs (SHIV-NI) were protected from a challenge with a heterologous pathogenic SHIV (Enose et al., 2002; Ui et al., 1999). Since live-attenuated virus vaccines mimic natural exposure to infectious disease, these vaccines effectively induce very durable humoral and cell-mediated immunity. Live-attenuated SIV/SHIVs have been shown to be effective vaccines in macaque models. Although safety considerations have limited trials of live-attenuated HIV vaccines in humans (Baba et al., 1999; Gundlach et al., 2000), determining the mechanism of protection of live-attenuated

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lentiviruses in primates may help to develop HIV-1 vaccines (Koff et al., 2006; Miller and Abel, 2005). In general, the immunogenicity of live-attenuated vaccines tends to increase with increasing virulence (Johnson and Desrosiers, 1998). Therefore, in attenuating a live virus, there is a trade-off between safety and immunogenicity. One promising new strategy to improve immunogenicity of live-attenuated vaccines is to genetically engineer a virus to co-express an immunostimulatory agent such as a cytokine adjuvant. Several studies have demonstrated that insertion of a cytokine in gene-deleted live-attenuated SIV/SHIVs could boost its immunogenicity and enhance its protection ability (Giavedoni et al., 1997; Stahl-Hennig et al., 2003). This would make it possible to obtain a higher level of immunogenicity from safer, less virulent strains.

Chemokines are key players in eliciting immune responses against viral infections, by selective activation of a subpopulation of immune cells and by attracting these cells to the site of infection (Luster, 1998). Regulated-on-activation-normal-T-cell-expressed-and-secreted (RANTES) is a CC-chemokine and a natural ligand for CC-chemokine receptors (CCRs) 1, 3, 4, and 5. CCR5, the main receptor of RANTES, is expressed mainly on subsets of monocytes, macrophages, NK cells, and T lymphocytes that are predominantly associated with T helper type-1 (Th1) responses (Bonecchi et al., 1998; Loetscher et al., 1998; Sallusto et al., 1998). An immune response polarized toward a more Th1 response is associated with a reduced viral load and non-progression of disease in HIV-1 infection (Imami et al., 2002). RANTES has been found to enhance cellular immune responses resulting in a more effective immune-modulating effect against HIV-1-related virus in rodent and monkey models (Frauenschuh et al., 2004; Kim et al., 1998; Waterman et al., 2004; Xin et al., 1999). In addition, infection of macaques with a live-attenuated SIV induced the production of CC-chemokines, and the up-regulation of CC-chemokines was found to be associated with the sterilizing immunity generated by the vaccine (Ahmed et al., 1999; Gauduin et al., 1999; Heeney et al., 1998). Moreover, RANTES has been shown to directly inhibit HIV-1 replication *in vitro* (Alkhatib et al., 1996; Cocchi et al., 1995). RANTES blocks or down-modulates CCR5 *in vitro*, which leads to suppression of CCR5-tropic (R5-tropic) HIV-1 infections (Proudfoot et al., 1999). These results make RANTES an attractive candidate as an immune adjuvant.

In order to test the adjuvant effects of RANTES in SHIV-macaque models, we previously genetically engineered a SHIV to co-express the human RANTES gene (SHIV-RANTES) and characterized its properties *in vitro* (Shimizu et al., 2006). In a previous *in vitro* study, along with the replication of SHIV-RANTES, RANTES was detected in the culture supernatant at a maximum level of 98.5 ng/ml in SHIV-RANTES-infected human $CD4^+$ M8166 cells. The expressed RANTES down-regulated the expression of CCR5 on PM1 cells, and suppressed the replication of BaL, a R5-tropic HIV-1.

In this study, we demonstrate that SHIV-RANTES provided effective cellular immunity in inoculated monkeys by inducing an antigen-specific proliferation of lymphocytes and by increasing an antigen-specific gamma interferon (IFN-) enzyme-linked immunospot (ELISpot) response.

Results

Immunization of SHIV-NI and SHIV-RANTES

A recombinant SHIV was engineered to express RANTES (SHIV-RANTES) in place of *nef* in SHIV-NI (Fig. 1A). To investigate the *in vivo* properties of a SHIV-RANTES, four rhesus monkeys (MM426, MM427, MM430, and MM431) were intravenously inoculated with 10^5 tissue culture 50% infectious dose ($TCID_{50}$) of SHIV-RANTES, and three animals (MM428, MM432, and MM434) were inoculated with 10^5 $TCID_{50}$ of SHIV-NI as a control. All monkeys were viremic within 2 weeks post inoculation (wpi) (Fig. 2A). The SHIV-NI and SHIV-RANTES inoculation induced systemic infection with similar peak plasma viral RNA (vRNA) levels, reaching 10^3 to 10^4 RNA copies/ml at 2 to 4 wpi and falling to almost below the detection limit by 8 wpi (Figs. 2A and B). This was also the case for two monkeys (MM346 and MM349) that were inoculated with the same dose of SHIV-NI in a previous report (Shimizu et al., 2005). The infectious viruses were transiently re-isolated at 1 to 6 wpi from co-culture of M8166 cells with PBMCs (data not shown). The stability of the RANTES gene in virus isolated from the vaccinated macaques was analyzed by

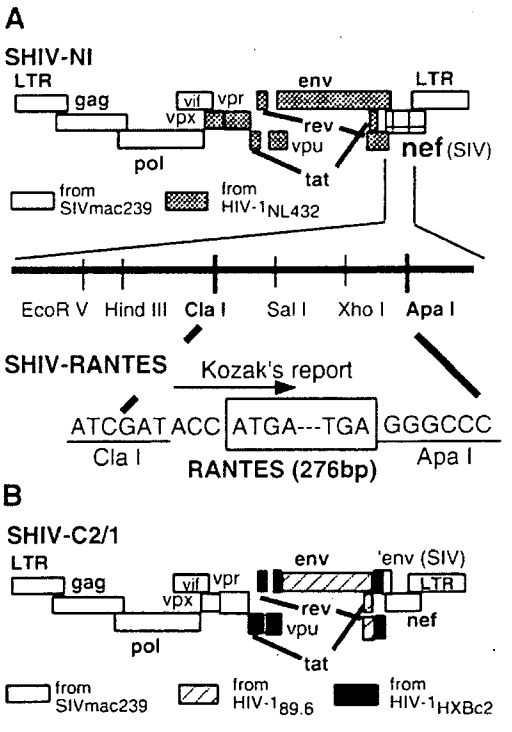

Fig. 1. Genetic structures of SHIVs used in this study. (A) The parental SHIV-NI and SHIV-RANTES. SHIV-NI has some unique restriction enzyme sites in place of the *nef* gene of SHIV-NM3rN. SHIV-NM3rN was constructed from HIV-1 NL432 (shaded regions) and SIV mac239 (white regions). The *ClaI* and *ApaI* region of SHIV-NI was replaced by the human RANTES gene (Shimizu et al., 2006). (B) The challenge virus SHIV-C2/1. SHIV-C2/1 (GenBank accession number AF217181) was generated by *in vivo* passage of SHIV-89.6 through cynomolgus monkeys (Shinohara et al., 1999). SHIV-89.6 was constructed from HIV-1 HXBc2, HIV-1 89.6, and SIV mac239.

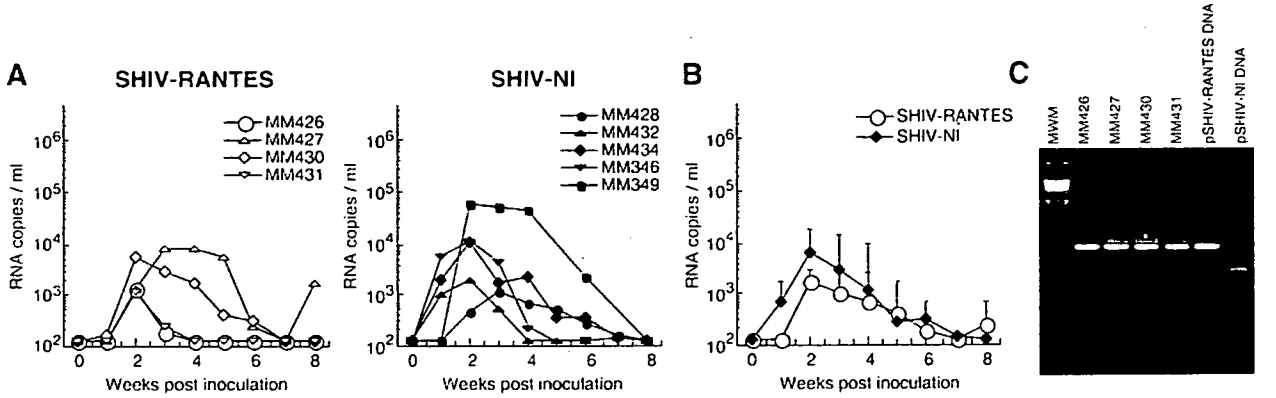

Fig. 2. Viral loads of SHIVs and RANTES gene stability. (A) Viral RNA loads in plasma of monkeys inoculated with SHIV-RANTES and SHIV-NI. Data for MM346 and MM349 are from a previous report (Shimizu et al., 2005). Plasma viral RNA loads were measured by RT-PCR. The detection limit of this assay was 1.2×10^2 copies/ml. (B) The geometric mean of viral RNA loads detected in the animals inoculated with SHIV-RANTES (white) and SHIV-NI (black) of each time point is reported. Values represent the mean with the standard error. (C) Stability of the inserted RANTES gene in SHIV-RANTES, as shown by PCR. DNA plasmids of SHIV-RANTES (pSHIV-RANTES DNA) and SHIV-NI (pSHIV-NI DNA) were used as the templates of the control. MWM, molecular weight markers of -*Hin*dIII.

PCR. The viruses that were re-isolated from SHIV-RANTES-infected monkeys kept the full-length of the inserted RANTES gene (Fig. 2C).

Lymphocyte phenotyping of PBMCs

After the infection, the number of peripheral $CD4^+$ lymphocytes remained within the normal level in both the SHIV-RANTES- and SHIV-NI-inoculated monkeys (Fig. 3A) and all monkeys clinically remained healthy. Thus, the pathogenicity of SHIV-RANTES and SHIV-NI infection to macaque monkeys was not significantly different. No significant differences were observed between the SHIV-NI- and SHIV-RANTES-inoculated monkeys in the cell surface markers of PBMCs (data not shown).

Expression of RANTES

In SHIV-NI-inoculated monkeys, plasma RANTES levels were about 2 to 3 ng/ml (Fig. 3B) by enzyme-linked immunosorbent assay (ELISA) throughout the observation period, which is similar to those in the parental SHIV-NM3rN-inoculated animals (Kwofie et al., 2000). The plasma RANTES levels in the SHIV-RANTES-infected monkeys were similar in SHIV-NI-infected monkeys after the infection. Due to the limited and local nature of viral replication, we could not detect the up-regulation of plasma level of RANTES in animals inoculated with SHIV-RANTES.

Secondary lymphoid tissues are the major sites of replication of HIV-1-related viruses. Thus, the expressions of RANTES and several cytokines mRNAs in inguinal lymph nodes from virus-inoculated monkeys were measured by real-time RT-PCR. Of note, the mRNA levels of RANTES in the lymph nodes were modestly higher in SHIV-RANTES-inoculated animals compared to control SHIV-NI-inoculated animals at 2 wpi (Fig. 3C), whereas the plasma viral loads were about the same in the two groups. Levels of IFN- , as a marker for Th1-type cytokines, and tumor necrosis factor alpha (TNF-) mRNAs were higher in SHIV-RANTES-immunized monkeys than in SHIV-NI-immunized monkeys. Interleukin-10 (IL-10) and IL-4 mRNA

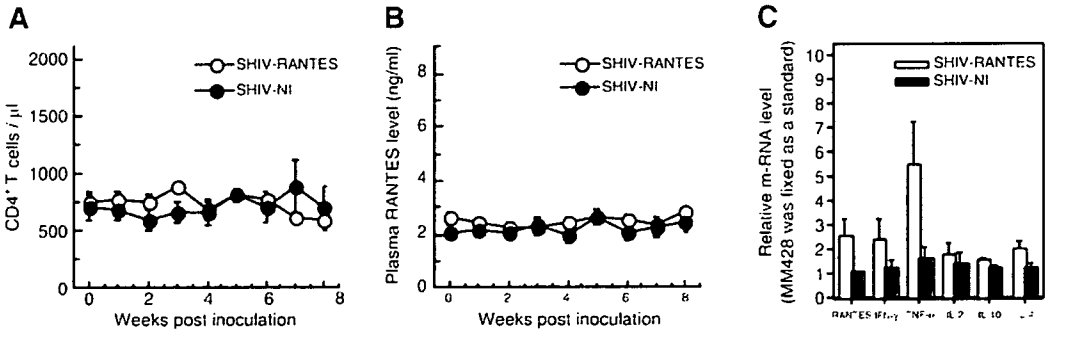

Fig. 3. Various markers in systemic and local sites in rhesus monkeys after inoculation with SHIV-RANTES (white) and SHIV-NI (black). (A) Time course of $CD4^+$ T cell counts. Values were calculated from the percentage $CD3^+CD4^+$ cells measured by flow cytometry. The mean $CD4^+$ T cell counts of animals inoculated with SHIV-RANTES (MM426, MM427, MM430, MM431) and SHIV-NI (MM428, MM432, MM434, MM436, MM349) at each time point. Values represent the mean with the standard error. (B) Time course of the mean of plasma RANTES levels measured by ELISA. Values represent the mean with the standard error. (C) Cytokines and chemokine mRNA levels in lymph nodes. MM428 was defined as a standard because it shows representative data. Data were expressed as the relative cytokine and chemokine mRNA levels calculated by the C_T method. Values represent the mean and standard error.

levels, as markers for Th2-type cytokines, and IL-2 mRNA levels were almost similar in the two groups. The proviral DNA loads in the lymph nodes from SHIV-RANTES-infected monkeys were similar to those in the SHIV-NI-infected monkeys (data not shown).

Antigen-specific CD4⁺ Th cell-proliferative response

Chemokines activate polyclonal and antigen-specific helper T cells during the induction of an immune response (Taub et al., 1996). To determine whether inoculation of the SHIV-RANTES elicited antigen-specific lymphocytes in the animals, proliferative responses to SIV Gag were measured with PBMCs by uptake of 5-bromo-2 -deoxyuridine (BrdU). In this study, antigen-specific T cell proliferation was induced especially in the SHIV-RANTES-inoculated monkeys (example shown in Fig. 4A). Some SHIV-RANTES-inoculated monkeys demonstrated polyclonal T cell proliferation even without stimulation, but the stimulation index (SI) of these monkeys showed a significant antigen-specific proliferative response (SI>2.0). Proliferative responses to Gag in the SHIV-RANTES group were observed as early as 2 wpi (Fig. 4B), while the proliferative responses to a SIV Gag were low in the SHIV-NI-inoculated monkeys. These results show that antigen-specific T cell proliferation was induced markedly in the SHIV-RANTES group after the infection.

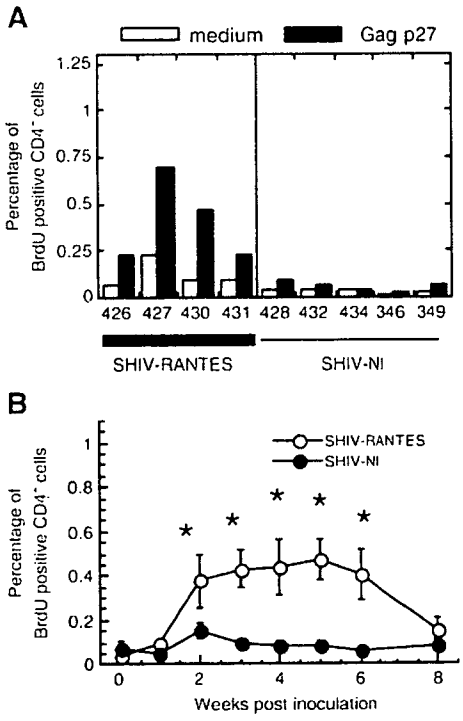

Fig. 4. Antigen-specific T cell proliferation responses. (A) Percentage of the SIV Gag-specific proliferated $CD4^+$ cells (solid bars) and unstimulated cells (open bars) at 6 wpi, as measured by BrdU uptake. (B) Kinetics of SHIV-specific Th cell proliferation in SHIV-RANTES- and SHIV-NI-inoculated rhesus monkeys. Values represent the mean and standard error. Asterisks (*) indicate statistically significant differences ($P<0.05$) as determined by the Mann–Whitney U test.

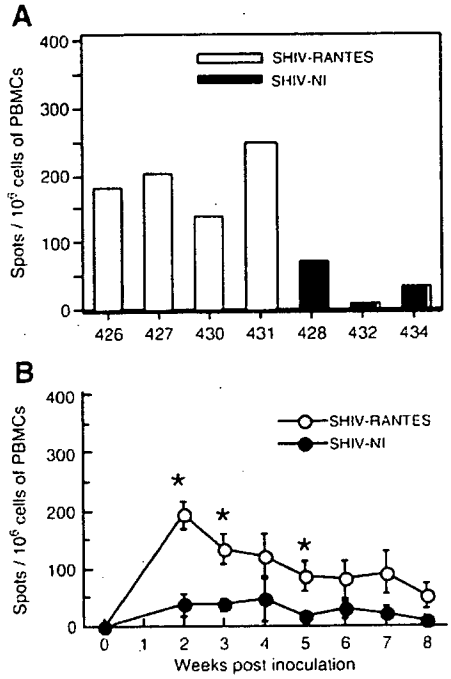

Fig. 5. Antigen-specific IFN- ELISpot responses. (A) Number of IFN-secreting PBMCs detected by ELISpot after stimulation with SIV Gag at 2 wpi. Graph bars represent the mean number of spots per million cells detected in duplicate cultures of PBMCs cultured with SIV Gag, after subtracting the mean number of spots found in duplicate control cultures of PBMCs in medium alone. (B) Kinetics of the SIV-Gag-specific IFN- ELISpot responses. Values represent the mean and standard error. Asterisks (*) indicate statistically significant differences ($P<0.05$) as determined by the Mann–Whitney U test.

Vaccine-induced SHIV-specific IFN- ELISpot

Two weeks after the immunizations, responses of SIV-Gag-specific IFN- ELISpot responses were strong in macaques immunized with SHIV-RANTES, weak in the two of the three macaques that had been immunized with SHIV-NI, and modest in the third (MM428) (example shown in Fig. 5A). The difference in the ELISpot results between SHIV-RANTES and SHIV-NI groups is statistically significant ($P<0.05$). In the SHIV-RANTES-inoculated monkeys, the antigen-specific IFN-ELISpot intensity peaked at 2 wpi and then returned to the normal low level by 8 wpi (Fig. 5B).

Peptide-based IFN- ELISpot assays were shown to be a reliable way of assessing $CD8^+$ cytotoxic T lymphocytes (CTL) responses (Schmitel et al., 1997). As no peptides to Gag were available in our laboratory, we used the protein in this assay. In order to establish whether $CD4^+$ Th1 cells or $CD8^+$ CTL were responsible for the IFN- production seen in the ELISpot assay, the SIV-Gag-specific IFN- ELISpot responses using $CD8^+$-depleted PBMCs obtained from monkeys were also assessed. Although the ELISpot responses varied among the monkeys, the responses were sustained in PBMCs that were depleted of $CD8^+$ cells (data not shown). This suggests that the antigen-specific IFN- responses seen in this study were, at least partly mediated by $CD4^+$ Th1 cells.

Antibody titer

To assess the humoral immune responses in the SHIV-RANTES and SHIV-NI-immunized monkeys, HIV-1 Env-specific antibodies (Ab) in the plasma were measured by the particle agglutination method. The levels of SHIV-specific Abs began to increase in all the infected animals at 3–5 wpi (Fig. 6). The kinetics and magnitude of the anti viral Ab response during the first 6 weeks after vaccination were similar in the two groups, suggesting that the initiation of the virus-specific Abs was not different in the two groups. However, in the next 2 weeks, the Ab titers in the SHIV-NI-infected animals continued to increase (Fig. 6B), whereas the Ab titers in the three of fours SHIV-RANTES-infected animals remained unchanged or gradually declined (Fig. 6A), resulting in a significant difference in Ab titers at 8 wpi ($P<0.05$).

Challenge with a pathogenic SHIV

To evaluate the protective effects provided by SHIV-RANTES, monkeys were challenged intravenously with 10^5 $TCID_{50}$ of heterologous pathogenic SHIV-C2/1 KS661 (Fig. 1B) at 8 wpi. As controls, four naive monkeys (MM298, MM299, MM338, and MM339) were infected intravenously with the same dose (10^5 $TCID_{50}$) of SHIV-C2/1 KS661. All of the control SHIV-C2/1-inoculated rhesus monkeys showed a pronounced decrease of $CD4^+$ T lymphocytes, reaching less than 50 cells/ l (Fig. 7A). The vRNA in the plasma of the control monkeys increased to above 10^7 copies/ml at 1 wpi, and remained at a high level (Fig. 7B).

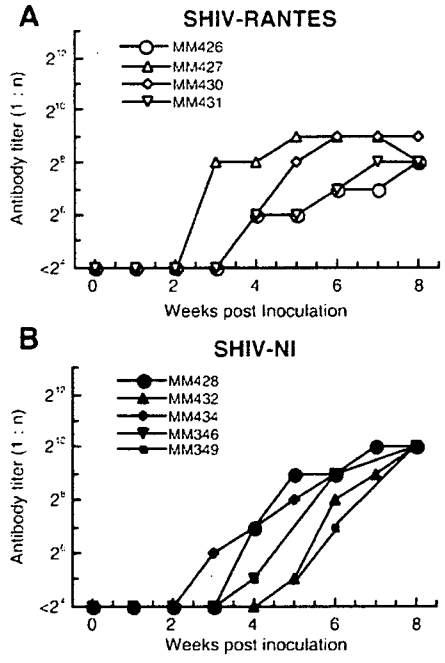

Fig. 6. Humoral immune responses, as expressed by anti-HIV-1 antibody titer. Antibody titers in SHIV-RANTES- (A) and SHIV-NI- (B) inoculated rhesus monkeys were analyzed by particle agglutination assay and are expressed as the maximum dilution of plasma to give a positive result.

After the challenge with a pathogenic SHIV-C2/1, the circulating $CD4^+$ T cell counts were maintained in all SHIV-RANTES-immunized monkeys (Fig. 7A). The peak plasma vRNA loads in the SHIV-RANTES group (10^4 and 10^5 copies/ml) were three orders of magnitude lower than those of the naive SHIV-C2/1 KS661-inoculated monkeys (Fig. 7B). The plasma virus loads in three of the four SHIV-RANTES-immunized monkeys rapidly declined to almost below the detection limit at 3 weeks post challenge (wpc). The peak plasma vRNA load of one of the SHIV-NI-immunized monkeys (MM349: 10^7 copies/ml) was similar to that of the control monkeys. The circulating $CD4^+$ T cell count in MM349, a SHIV-NI-immunized monkey, remained within the normal range in spite of a transient but intense viremia in the plasma (Fig. 7A). There was no difference in the nature of the $CD4^+$ T cell levels and plasma viral RNA loads between SHIV-NI- and SHIV-RANTES-immunized animals.

Post-challenge anti-SHIV immune responses

We next analyzed the anamnestic cellular and humoral immune responses against SHIV-C2/1 challenge. At the time of challenge, similar proportions of SHIV-RANTES- and SHIV-NI-vaccinated animals had SIV-Gag-specific T cell-proliferative responses in PBMC. At 1 wpc, all SHIV-RANTES-vaccinated animals had detectable anti-SHIV-specific T cell-proliferative responses, while none of the SHIV-NI vaccinated-animals showed such responses (Fig. 8A). At 2 wpc, antigen-specific T cell proliferative responses dramatically increased in the SHIV-RANTES-vaccinated monkeys and only moderately increased in the SHIV-NI vaccinated-animals. These results suggest that the $CD4^+$ Th cell proliferation was greatly enhanced in the SHIV-RANTES-immunized group.

The enhancements of post-challenge anti-SHIV IFN- ELISpot responses were also observed in PBMCs from the SHIV-RANTES-immunized group as early as 1wpc (Fig. 8B). Similarly in SHIV-RANTES-vaccinated animals, SHIV-specific ELISpot responses in all SHIV-NI vaccinated-animals also could be detected at week 2–3 post-challenge, but the magnitude of IFN- ELISpot responses in SHIV-NI-vaccinated animals was less than that in the SHIV-RANTES-vaccinated monkeys. After the post-challenge, the ELISpot response was markedly higher in the SHIV-RANTES-immunized group than in the SHIV-NI group ($P<0.05$).

As for humoral immune response, after the pathogenic SHIV-C2/1 challenge, the Ab titers of the SHIV-NI-vaccinated animals and two of the four SHIV-RANTES-vaccinated animals rose rapidly, while the titers of the other two SHIV-RANTES-vaccinated animals rose moderately (data not shown).

Quantification of proviral DNA after the pathogenic challenge

To examine the virus clearance in the animals, the proviral DNAs in the various tissues of the monkeys after the SHIV-C2/1 challenge were measured by quantitative PCR for SHIV-C2/1-specific SIV *vpr* regions. Of note, the copy numbers of the challenge viruses in the tissue samples from the SHIV-RANTES-

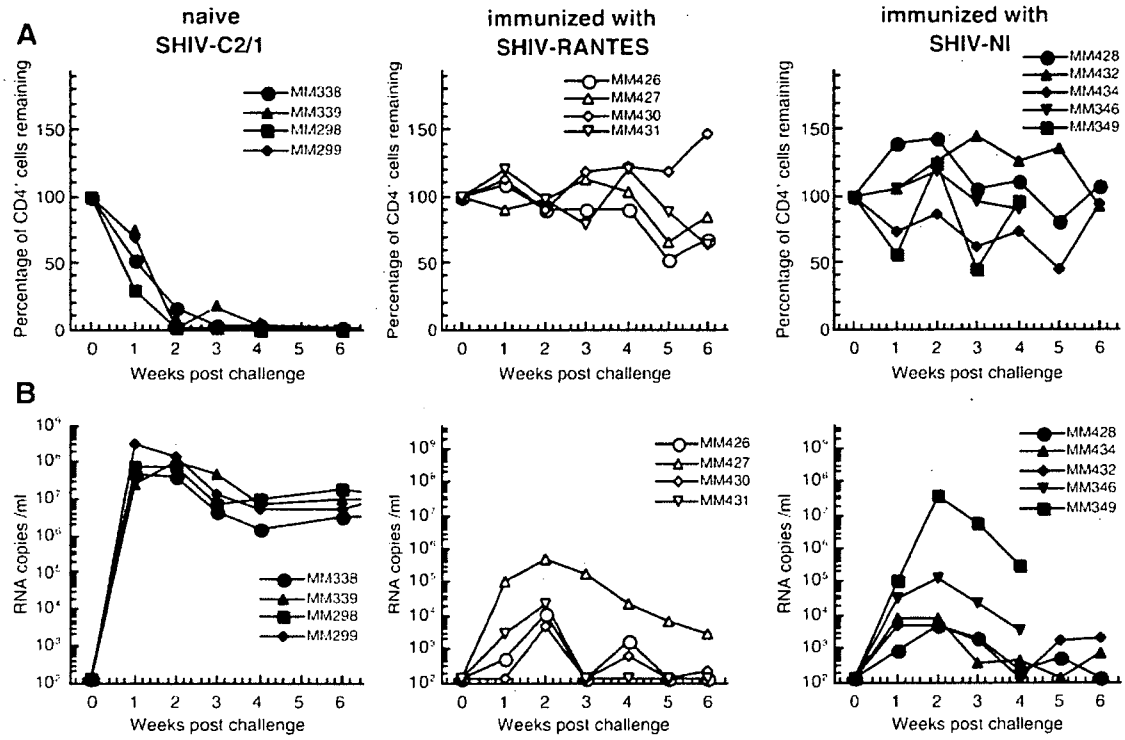

Fig. 7. Change in the number of circulating $CD4^+$ T cells (A) and SHIV-C2/1-specific viral RNA loads (B) of the SHIV-NI- and SHIV-RANTES-immunized monkeys after SHIV-C2/1 challenge. Values are expressed as a percentage of the pre-challenge values of each monkey. Plasma viral RNA loads after the heterologous pathogenic SHIV (SHIV-C2/1) challenge were measured by RT-PCR. The detection limit of this assay was 1.2×10^2 copies/ml. Left panels show results for naive SHIV-C2/1-inoculated monkeys (MM338, MM339, MM298, and MM299) (Shimizu et al., 2005).

immunized monkeys were lower than those in the SHIV-NI-immunized monkeys (Fig. 9A), although the difference was not statistically significant. For examples, proviral DNAs in the thymus of the three of four SHIV-RANTES-immunized monkeys were below the detection limit (<5 copies/ g of total cellular DNA), while proviral DNAs in the thymus of the SHIV-NI-immunized monkeys were ranging from 1.2×10^1 to 1.3×10^3 copies/ g of total cellular DNA (Fig. 9B). The proviral DNA load (1.6×10^2 copies/ g of total cellular DNA) in the remaining SHIV-RANTES-immunized monkey, MM427, was similar to that of the SHIV-NI-immunized monkeys (Fig. 9B). These results show that immunization of SHIV-RANTES may have immediately eliminated the heterologous challenge virus from the organs of some monkeys.

Discussion

There is much hope that the worldwide spread of HIV-1 will be controlled by the development of an HIV-1 vaccine. The expression of an inserted-cytokine or chemokine gene from a genetically engineered vaccine virus provides adjuvant effects locally at the site of virus replication. Several studies have demonstrated that insertion of a cytokine in a gene-deleted live-attenuated SIVs could boost the immunogenicity of the viruses and enhance its protection ability (Giavedoni et al., 1997; Stahl-Hennig et al., 2003). Although the concept of introducing a modified live virus as a vaccine is an old one, the concept of genetically engineering a virus to co-express an anti-viral agent should be further examined. Chemokines are secreted during the generation of an immune response. Release of RANTES has been reported to enhance the function of HIV-1-specific CTLs. As an example of the immune adjuvant effect, RANTES enhanced cellular immune responses resulting in a more effective immune-modulating effect against HIV-1 in the rodent and monkey models (Frauenschuh et al., 2004; Kim et al., 1998; Waterman et al., 2004; Xin et al., 1999). The aim of this study was to clarify the adjuvant effect of RANTES against HIV-1-related virus vaccine candidate in a SHIV-macaque model. For this purpose, we have genetically engineered a *nef*-deleted attenuated SHIV to express the RANTES gene, so that when viral transcription occurs, RANTES will also be produced locally.

The inoculation of SHIV-RANTES induced a remarkable antigen-specific $CD4^+$ Th cell-proliferative response. Virus-specific T helper responses play an important role in maintaining effective immunity such as CTL responses against viral pathogens (Kalams and Walker, 1998; Matloubian et al., 1994). Antigen-specific T cell proliferation is important for the control of AIDS pathogenesis. HIV-1-specific $CD4^+$ T cell-proliferative responses have been shown to be inversely related to viral loads and were initially only observed in long-term nonprogressors and in individuals who were treated during acute HIV-1 infection (Rosenberg et al., 1997, 2000). In mice models, co-injection of RANTES-expressing plasmids with

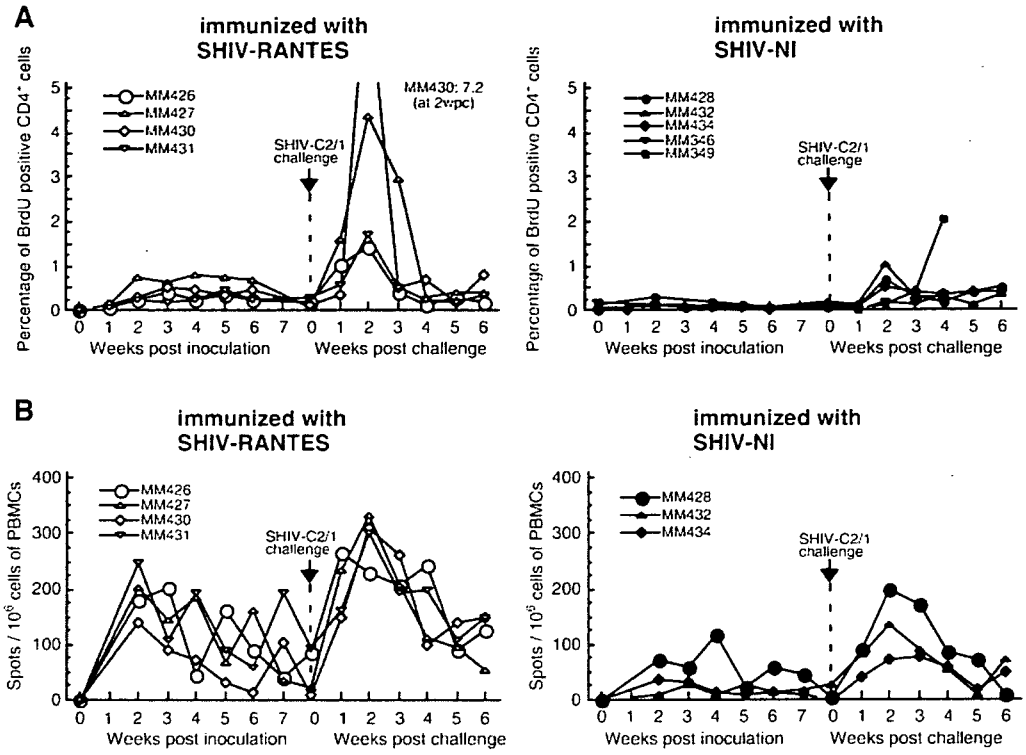

Fig. 8. Post-challenge antigen-specific immune responses. (A) Antigen-specific T cell proliferation responses after SHIV-C2/1 challenge. Kinetics of SHIV-specific Th cell proliferation in SHIV-RANTES- and SHIV-NI-inoculated rhesus monkeys after SHIV-C2/1 challenge. (B) Antigen-specific IFN- ELISpot responses after SHIV-C2/1 challenge. Kinetics of the SIV-Gag-specific IFN- ELISpot responses in SHIV-RANTES- and SHIV-NI-inoculated rhesus monkeys after SHIV-C2/1 challenge.

HIV-1 Env DNA vaccines enhanced antigen-specific splenocyte proliferation (Frauenschuh et al., 2004; Kim et al., 1998). Our results suggest that the co-expression of viral antigen and RANTES stimulates antigen-specific T cell proliferation, which may help to increase the antiviral immune response. Similarly, inoculation with SHIV-RANTES resulted in dramatic changes in the levels of antigen-specific IFN- ELISpot response. Many studies have shown that chemokine RANTES enhanced cellular immune responses resulting in a more effective immune-modulating effect against HIV-1-related virus. Although some virus-specific immune responses were also elicited in the SHIV-NI-immunized monkeys; combining the adjuvant RANTES with a live-attenuated SHIV had a dramatic effect on the cellular immune responses.

As live-attenuated vaccines, both SHIV-RANTES and SHIV-NI elicited strong humoral immune responses in monkeys. Of note, Ab titers in the SHIV-RANTES-vaccinated monkeys were lower than those in the SHIV-NI-vaccinated monkeys at the time of challenge. Many studies have reported that RANTES enhanced the cell-mediated immunity. However, the effect of RANTES on the humoral immune response is less clear, with some studies reporting a positive effect (Lillard et al., 2001; Xin et al., 1999), and others reporting either no effect (Kim et al., 1998) or a negative effect (Frauenschuh et al., 2004). In mice models, antibody levels against HIV antigen were lower in mice injected with plasmids encoding both RANTES and HIV antigen than in mice injected with plasmids encoding antigen alone (Frauenschuh et al., 2004). One possible explanation is that the combination of RANTES and antigen induced mainly a Th1-biased response, as indicated by a higher number of IFN- secreting cells, resulting in a lower antibody titers compared to vaccination with antigen alone (Frauenschuh et al., 2004).

Some studies have shown that RANTES enhanced replication of CXCR4 (X4)-tropic and R5X4-tropic HIV-1, both of which depend on a signal transduction and the enhanced HIV-1 replication is associated with increased colocalization of CD4 and CXCR4 (Kinter et al., 1998). Many studies have shown that RANTES and its analogues suppress the infection of R5-tropic lentiviruses *in vitro*. However, little information is available on the role of RANTES on X4-tropic lentivirus replication. The over-expression of RANTES may increase the replication of X4-tropic SHIV. A safety concern about live-attenuated viruses is that their replication rate may increase. In this regard, although SHIV-RANTES expressed a high amount of biologically active RANTES *in vitro*, we did not observe increased SHIV replication in either human or monkey cell lines (Shimizu et al., 2006). The present results show that both SHIV-NI and SHIV-RANTES replicated at low levels in the infected animals. Expression of RANTES by SHIV *in vivo* did not result in increased viral replication. SHIV vectors containing the RANTES gene were still attenuated and resulted in mild infection, and the infected animals were able to induce very effective immune responses.

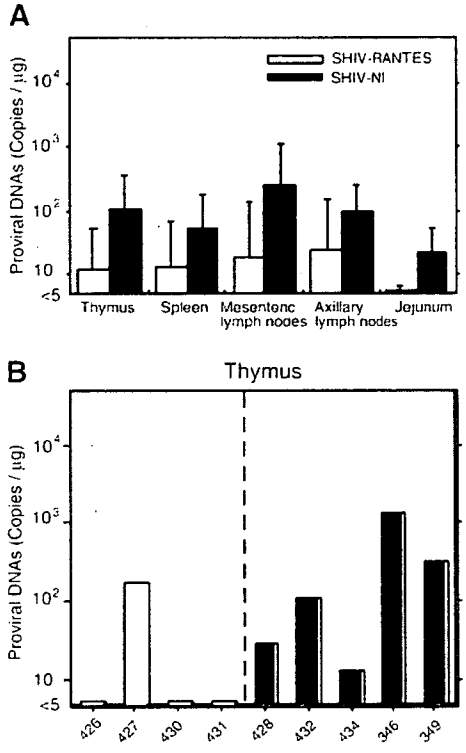

Fig. 9. Proviral DNA loads in various tissues of the SHIV-RANTES- and SHIV-NI-immunized monkeys after the pathogenic SHIV-C2/1 challenge. (A) The geometric mean of proviral DNA loads detected in the tissues from animals immunized with SHIV-RANTES (open bars) and SHIV-NI (solid bars) is reported. Values represent the mean with the standard error. (B) Proviral DNA loads in thymus of monkeys immunized with SHIV-RANTES and SHIV-NI. Proviral DNA loads of the challenge viruses were determined by quantitative PCR and are expressed as viral DNA copy numbers per microgram of total DNA extracted from the tissue homogenates. The detection limit of this assay was 5 copies/ g of total DNAs. Monkeys were euthanized at 5–6 wpc.

The immune responses induced by an attenuated virus can increase the immunity to a pathogenic virus. The monkeys immunized with the SHIV-NI were protected from a challenge with a heterologous pathogenic SHIV (Enose et al., 2002; Uji et al., 1999). Several studies have demonstrated that insertion of a cytokine in a gene-deleted live-attenuated SIV could boost its immunogenicity and enhance its protective ability (Giavedoni et al., 1997; Stahl-Hennig et al., 2003). In the present study, the monkeys that received the inoculation of SHIV-RANTES were partially protected from a challenge with a heterologous pathogenic SHIV-C2/1. It is important to mention that PBMCs obtained from SHIV-RANTES-immunized monkeys after SHIV-C2/1 challenge show robust cellular $CD4^+$ Th responses and ELISpot responses. A SHIV genetically engineered to express the RANTES gene enhanced the anamnestic immune responses against a heterologous pathogenic SHIV-C2/1 challenge, as well as the primary immune responses. However, there was no difference in the nature of the $CD4^+$ T cell levels and plasma viral RNA loads between SHIV-NI- and SHIV-RANTES-immunized animals, while the proviral DNA loads of the challenge virus in the some SHIV-RANTES-immunized monkeys were lower than those in the SHIV-NI-immunized monkeys. The control SHIV-NI, live-attenuated vaccine candidate also provided protection against pathogenic SHIV challenge in monkeys. Thus, the impacts on *in vitro* measures of cellular immunity do not predict *in vivo* protection, in large part because the *nef*-deleted SHIV is an effective immunogen. The SHIV-NI-vaccinated monkeys were partially protected despite the fact that antiviral immune responses were much weaker after the vaccination than they were in the SHIV-RANTES-immunized monkeys. The magnitude of virus-specific $CD4^+$ T cell responses augmented by RANTES in the SHIV-RANTES-immunized monkeys was not sufficient to improve the efficacy of the parental vaccine. Either greater immune augmentation or additional antiviral immune responses such as $CD8^+$ T cells and neutralizing antibodies may be needed to truly improve the efficacy of their parental vaccine.

In conclusion, our results suggest that the addition of RANTES to the SHIV had measurable effects on immunogenicity but still did not appear to afford greater protection against pathogenic SHIV challenge compared to control SHIV-NI-immunized animals. Additional work is needed to improve the magnitude and/or quality of these responses to achieve a measurable effect on vaccine efficacy.

Materials and methods

Viruses

SHIV-NM3rN, having HIV-1 NL432 genes on a SIVmac239 background, was utilized as a starting material. The SHIV-*nef* vector designated as SHIV-NI has some restriction enzyme sites in place of the *nef* gene of SHIV-NM3rN (Kuwata et al., 2000). SHIV-NI did not down-modulate $CD4^+$ cells, and did not cause AIDS like disease in macaques. The human RANTES gene was inserted into SHIV-NI to generate SHIV-RANTES (Fig. 1A). The source of the *env* gene of SHIV-RANTES was a X4-tropic virus HIV-1 NL432. *In vitro*, RANTES production by SHIV-RANTES did not limit replication of the virus (Shimizu et al., 2006). Virus stocks were prepared with the $CD4^+$ human T cell line M8166 (a subclone of C8166).

A heterologous pathogenic SHIV-C2/1 KS661 was used for the challenge virus (Fig. 1B). SHIV-C2/1 KS661 is a molecular clone derived from SHIV-C2/1 (GenBank accession number AF217181), and causes rapid $CD4^+$ T cell depletion (Shinohara et al., 1999). The *env* gene of SHIV-C2/1 was derived from HIV-1 89.6, and is antigenically different from the *env* gene of SHIV-NI and SHIV-RANTES.

Immunization and challenge

Male rhesus macaques (*Macaca mulatta*) were cared for at the Institute for Virus Research, Kyoto University in accordance with regulations approved by the Institutional Animal Care and Use Committee of the Institute for Virus Research, Kyoto University. Seven animals were divided into two groups: SHIV-RANTES (MM426, MM427, MM430, and MM431; $n=4$) and

SHIV-NI (MM428, MM432, and MM434; $n=3$). Monkeys were inoculated intravenously with 10^5 $TCID_{50}$ of each virus. At 8 wpi, all animals were challenged intravenously with a 10^5 $TCID_{50}$ of pathogenic SHIV-C2/1 KS661. Citrate-anticoagulant blood samples were collected under anesthesia with ketamine hydrochloride. Blood samples were phenotypically characterized on a FACSCaliber flow cytometer (Becton Dickinson, San Jose, CA) and were separated into plasma and peripheral blood mononuclear cells (PBMCs) by Ficoll density centrifugation (Nacalai Tesque, Inc. Kyoto, Japan). Inguinal lymph nodes were obtained from these animals by biopsy at 2 wpi and before infection. Single-cell suspensions were prepared from the inguinal lymph nodes with a 100 m nylon cell strainer (Becton Dickinson). Data from two monkeys (MM346 and MM349) that were inoculated with the same dose of SHIV-NI in a previous report (Shimizu et al., 2005) were also utilized. Data from four naive monkeys (MM298, MM299, MM338, and MM339) that were infected intravenously with the same dose (10^5 $TCID_{50}$) of SHIV-C2/1 KS661, were used as control (Shimizu et al., 2005).

Lymphocyte immunophenotyping

Anticoagulated whole-blood specimens or lymphocytes obtained from peripheral lymph node biopsies were immunophenotyped with the following monoclonal antibodies (MAbs): anti-CD4 (Nichirei, Tokyo, Japan), anti-CD8 (Becton Dickinson), anti-CD3 (BioSource International), anti-CD20 (Becton Dickinson), anti-CD14 (Beckman Coulter, Miami, FL), anti-CCR5 (Pharmingen, San Diego, CA), anti-CXCR4 (Pharmingen), anti-CD28 (Pharmingen), anti-CD95 (Pharmingen), anti-CD69 (Pharmingen), anti-CD80 (Pharmingen), anti-CD154 (Pharmingen), anti-CD212 (Pharmingen), and anti-HLA-DR (Pharmingen). Ten thousand events per sample were acquired by the FACSCaliber, and data were analyzed by CellQuest software (Becton Dickinson) and FlowJo software (TreeStar, San Carlos, CA). Absolute lymphocyte counts on blood specimens were obtained with an automated hematology analyzer (F-820: Sysmex, Kobe, Japan). Peripheral blood $CD4^+$ T lymphocytes counts were calculated by multiplying the total lymphocyte count by the percentage of $CD3^+CD4^+$ T cells.

Plasma viral RNA loads

Plasma viral RNA loads were determined by quantitative RT-PCR as described previously (Enose et al., 2002; Suryanarayana et al., 1998). Total RNAs were prepared from plasma with a QIAamp viral RNA kit (QIAGEN, Valencia, CA), and RT-PCR was performed using a Platinum Quantitative RT-PCR ThermoScript one-step system kit (Invitrogen Corp., Carlsbad, CA). The plasma viral loads of SHIV-NI, SHIV-RANTES, and the challenge virus were differentially evaluated with primer pairs specific to SHIV-NM3rN and SHIV-C2/1, respectively (Enose et al., 2004). These reactions were performed with a Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) and analyzed using the manufacturer's software.

Virus isolation

To isolate the virus, $CD8^+$-depleted PBMCs were co-cultured with M8166 cells, as described previously (Enose et al., 2004). $CD8^+$-depleted PBMCs were obtained from each monkey by using mouse anti-human CD8 MAb (Nichirei, Japan) and sheep anti-mouse IgG magnetic beads (Dynabeads M-450; Dynal A. S., Oslo, Norway). Viral recovery, based on the syncytium formation, was monitored for 4 weeks.

Isolation of proviral DNA

Proviral DNA was extracted from 1×10^6 PBMCs and tissues of the inoculated monkeys. When the virus was re-isolated, $CD8^+$-depleted PBMCs, which were co-cultured with M8166 cells, were also monitored. Cellular DNAs were extracted using DNeasy tissue kits (QIAGEN). To check the stability of inserted RANTES gene in SHIV-RANTES, the proviral DNA fragments covering the inserted RANTES gene in SHIV-RANTES were amplified by PCR with primers specific for the *nef* region (Kuwata et al., 2000; Shimizu et al., 2006). The lengths of DNA fragments generated by this reaction were 415 bp for the SHIV containing the intact RANTES gene and 154 bp for SHIV-NI. The proviral DNA loads were determined by quantitative PCR. PCR was performed with a Taqman PCR reagent kit (Perkin Elmer) using the same primer and probe which were used in RT-PCR. A standard curve was generated from a plasmid DNA sample containing the full genome of SHIV-NM3rN or SHIV-C2/1 KS661, which was quantified with a UV-spectrophotometer.

Determination of plasma level of RANTES

Plasma RANTES concentration was measured using a human RANTES ELISA kit (R&D Systems, Inc., Minneapolis, MN), which is known to cross-react with rhesus RANTES (Kwofie et al., 2000). Because chemokine gene transcription can change dramatically during storage of whole blood for periods of as little as 6 h (Tanner et al., 2002), platelet-poor plasma was prepared from whole blood samples within 2 h by centrifugation at 10,000×*g* for 10 min at 4 °C according to the manufacturer's recommendation. Samples that had been aliquoted and stored at −80 °C were thawed immediately before assay and were not reused.

Chemokine and cytokine mRNA analysis by real-time PCR amplification

Inguinal lymph nodes suspensions were collected from rhesus macaques at the time of biopsy. Total RNA was isolated using an RNA Mini kit (Qiagen). All samples were DNase (Qiagen)-treated for 20 min at 37 °C. cDNA was prepared using random hexamer primers (Invitrogen) and M-MLV-Reverse-Transcriptase (Super Script III, Invitrogen). Real-time PCR amplifications were conducted by SYBR Green methods. Oligonucleotide primers were designed for the TaqMan assay based on previously published reports (Abel et al., 2001; Hofmann-Lehmann et al., 2002). The following primers were

used: IFN- (F, GAAAAGCTGACCAATTATTCGGTAA; R, AGCCATCACTTGGATGAGTTCA), IL-2 (F, CACCAGGATGCTCACATTTAAGTT; R, GAGGTTTGAGTTCTTCTTCTAGACACTGA), IL-4 (F, AAACGGCTCGACAGGAACCT; R CTCTGGTTGGCTTCCTTCACA), IL-10 (F, CACGACCCAGACATCAAGGA; R, CCACGGCCTTGCTCTTGTT), TNF- (F, GGCTCAGGCAGTCAGATCATC; R, GCTTGAGGGTTTGCTACAACATG), RANTES (F, ACCAGTGGCAAGTGCTCCA; R, TGGCACACACTTGGCGATT), and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (F, GCACCACCAACTGCTTAGCAC; R, TCTTCTGGGTGGCAGTGATG). Primers were used at a final concentration of 50 or 300 nM. The reaction was carried out in a 25 l reaction volume containing appropriate diluted 5 l of cDNA with SYBR Green PCR master Mix (Applied Biosystems). All sequences were amplified using the following amplification program: 2 min at 50 °C, 10 min at 95 °C, followed by 50 to 55 cycles of 15 s at 95 °C and 1 min at 60 °C with a Prism 7700 or 7000 Sequence Detector (Applied Biosystems). Samples were tested in duplicate, and the PCR for the housekeeping gene (GAPDH) and the target (cytokine or chemokine) gene was run in parallel on the same plate. The relative quantities of cytokine mRNA transcripts were determined by comparative threshold cycle (C_T) methods (C_T methods) (Pfaffl, 2001). In this analysis, the C_T value for the housekeeping gene is subtracted from the C_T value of the target gene (C_T). The target gene and the housekeeping gene were amplified with the same efficiency (data not shown). The mRNA samples from one SHIV-NI-immunized monkey, MM428, were defined as a standard. C_T values were calculated as follows: C_T= C_T value for each individual monkey- C_T value of standard monkey. Then, the relative quantitation of cytokine mRNA expression level was calculated by $2^{-\ C_T}$.

Proliferation assays

Lymphocyte proliferation was measured by incorporation of BrdU into the stimulated-lymphocytes (Shimizu et al., 2005). PBMCs (2×10^5) were cultured in a 96-well plate in RPMI 1640 with 10% FCS (complete RPMI medium). The recombinant viral proteins of SIV Gag (SIVmac251 p27; 5.0 g/ml; Advanced Biotechnologies, Inc., Columbia, MD) were used for antigen-specific stimulation. Concanavalin A (ConA; 0.5 g/ml; Sigma, St. Louis, MO) was used for polyclonal stimulation. The plates were incubated for 72 h at 37 °C. After the incubation, the cells were cultured for another 24 h in the presence of BrdU. Then lymphocyte proliferation was measured using a BrdU-Flow kit (Pharmingen) following the manufacturer's recommendations. To characterize the lymphocyte subsets in the proliferated cells, cells were stained for surface markers with MAb CD4-PE (Nichirei) and CD8-PerCP (Becton Dickinson). The cells were stained with MAb BrdU-FITC after fixation and permeabilization, and then analyzed by FACScan (Becton Dickinson). Proliferation responses to the antigens were considered positive if the stimulation index (SI = titers for antigen-stimulated samples/titers for control samples without antigens) exceeded 2.0 (Shimizu et al., 2005).

IFN- ELISpot

The frequency of antigen-specific IFN- -producing cells from PBMCs was determined by ELISpot analysis. The IFN- ELISpot assay was conducted according to the protocol provided by the manufacturer (Mabtech, Nacka Strand, Sweden). Briefly, 96-well Multiscreen-IP plates (Multiscreen MAIPS45-10, Millipore, Bedford, MA) were coated with 15 g/ml anti-IFN- Mab in sterilized phosphate-buffered saline (PBS) overnight at 4 °C. Then, PBMCs were incubated with or without 5 g/ml of SIV Gag for 24 h at 37 °C and were added to the pre-coated plates at 2.0 and 5.0×10^5 cells/well in complete RPMI medium. Experiments were performed in duplicate wells. PBMCs which were incubated with 0.5 g/ml of ConA for 2 h at 37 °C were also used as a polyclonal stimulation. Plates were incubated for about 36 h at 37 °C. Following incubation, cells were removed, and residual cells were lysed with ice-cold water for 10 min. Biotinylated anti-IFN- monoclonal antibody was added and incubated for 3 h at room temperature, followed by addition of streptavidin-horseradish peroxidase for 2 h at room temperature. Spots were developed with a Vector *NovaRED*™ Substrate kit for peroxidase (Vector Labs, CA). The number of spot-forming cells was determined by using a light microscope. The average number of spots present in the non-stimulated cultures was subtracted from the average number in the antigen-stimulated cultures.

Determination of anti-SHIV antibody titers

Anti-SHIV antibody titers in the plasma of the monkeys were determined using a commercial particle agglutination test kit (Genedia HIV-1/2, Fujirebio Inc., Tokyo, Japan). The samples were serially two-fold diluted and assayed following the manufacturer's recommendations. The end-point titer was determined as the highest dilution to give a positive result.

Statistical analysis

Differences between the SHIV-RANTES- and SHIV-NI-inoculated monkeys were analyzed with the Mann- Whitney *U* test. *P* values <0.05 were considered statistically significant.

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