

Rapid dissemination of a pathogenic simian/human immunodeficiency virus to systemic organs and active replication in lymphoid tissues following intrarectal infection

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A better understanding of virological events during the early phase of human immunodeficiency virus 1 (HIV-1) infection is important for development of effective antiviral vaccines. In this study, by using quantitative PCR and an infectious plaque assay, virus distribution and replication were examined in various internal organs of rhesus macaques for almost 1 month after intrarectal inoculation of a pathogenic simian immunodeficiency virus/HIV chimeric virus (SHIV-C2/1-KS661c). At 3 days post-inoculation (p.i.), proviral DNA was detected in the rectum, thymus and axillary lymph node. In lymphoid tissues, infectious virus was first detected at 6 days p.i. and a high level of proviral DNA and infectious virus were both detected at 13 days p.i. By 27 days p.i., levels of infectious virus decreased dramatically, although proviral DNA load remained unaltered. In the intestinal tract, levels of infectious virus detected were much lower than in lymphoid tissues, whereas proviral DNA was detected at the same level as in lymphoid tissues throughout the infection. In the thymus and jejunum, CD4CD8 double-positive T cells were depleted earlier than CD4 single-positive cells. These results show that the virus spread quickly to systemic tissues after mucosal transmission. Thereafter, infectious virus was actively produced in the lymphoid tissues, but levels decreased significantly after the peak of viraemia. In contrast, in the intestinal tract, infectious virus was produced at low levels from the beginning of infection. Moreover, virus pathogenesis differed in CD4 single-positive and CD4CD8 double-positive T cells.

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INTRODUCTION

A better understanding of virological events during the early phase of human immunodeficiency virus 1 (HIV-1) infection is essential for the development of effective vaccines for preventing virus transmission. This is especially true for mucosal infections, which are the major mode of HIV-1 transmission. Moreover, high virus load in the early phase of infection has been reported to correlate with earlier onset of AIDS (Fauci, 1996; Mellors *et al.*, 1995; Schacker *et al.*, 1996). Therefore, data obtained from the early phase of infection would help to define the pathogenesis of HIV-1.

Several non-human primate models have been used to investigate the early phase of HIV-1 infection (Joag *et al.*, 1997; Lu *et al.*, 1998). In some studies using macaques

inoculated with *Simian immunodeficiency virus* (SIV) or an SIV/HIV-1 chimeric virus (SHIV) by a mucosal route (i.e. oral, rectal or vaginal), the virus spread to the systemic lymphoid tissues within 3–7 days post-inoculation (p.i.) following replication for a period of time in the local region (Couëdel-Courteille *et al.*, 1999, 2003; Hirsch *et al.*, 1998; Spira *et al.*, 1996; Stahl-Hennig *et al.*, 1999). However, recent studies have shown that the virus can spread more rapidly to the systemic tissues. Hu *et al.* (2000) detected SIV-infected cells in draining lymph nodes within 18 h of intravaginal exposure. Milush *et al.* (2004) showed that SIV spread to systemic lymphoid tissues 1–2 days after oral inoculation. Miller *et al.* (2005) showed that the dissemination of SIV infection to systemic lymphoid tissues occurred within 1–3 days of vaginal inoculation, although virus production

at this site was established later. Furthermore, Veazey *et al.* (1998) reported that the intestinal tract was one of the major sites of SIV replication and CD4⁺ T cell depletion in the early phase of infection. In a study using SHIV, Harouse *et al.* (1999) suggested that SHIV using CCR5 as co-receptor for virus entry caused a dramatic loss of CD4⁺ intestinal T cells followed by a gradual depletion in peripheral CD4⁺ T cells, whereas infection with SHIV using CXCR4 caused a profound loss in peripheral T cells that was not paralleled in the intestine.

The goals of the present study were to investigate the distribution of pathogenic virus in systemic tissues early after mucosal infection and to determine whether these tissues produced infectious virus, which is considered to play a major role in the spread of virus in the body. A pathogenic molecular clone, SHIV-C2/1-KS661c (Shinohara *et al.*, 1999), which uses two major chemokine receptors, CCR5 and CXCR4, as co-receptors for virus entry, was used to inoculate rhesus macaque monkeys intrarectally. Proviral DNA and infectious virus were quantified by quantitative PCR and infectious plaque assay, respectively. Virus load in the infected individuals has usually been quantified by the copy number of virus RNA or DNA using PCR or by the immunodetection of core protein, p24 or p27 (Chun *et al.*, 1997; Sei *et al.*, 1994; Zhang *et al.*, 1999). However, these methods do not differentiate between infectious and non-infectious virus. The infectious plaque assay used in this study quantified infectious virus only (Kato *et al.*, 1998; Miyake *et al.*, 2004). Our results show that the virus spread rapidly to the systemic tissues soon after intrarectal infection. Thereafter, infectious virus was actively produced in the lymphoid tissues, but decreased significantly after the peak of viraemia. In the intestinal tract, lower levels of infectious virus were produced than in lymphoid tissues throughout the infection.

METHODS

Virus. SHIV-C2/1 was generated by *in vivo* passage of SHIV-89.6 (containing *env*, *tat*, *rev* and *vpu* derived from primary isolates of HIV-1) (Shinohara *et al.*, 1999). SHIV-C2/1-KS661c is a molecular clone constructed from the consensus sequence of SHIV-C2/1 (GenBank accession no. AF217181). SHIV-C2/1-KS661c can infect macaque monkeys by intravenous and intrarectal routes and cause precipitous viraemia and drastic CD4⁺ cell depletion. Virus stock was prepared from supernatant of a human lymphoid cell line, CEMx174, and stored in liquid nitrogen (−190 °C) until use. The TCID₅₀ of the virus stock was measured in CEMx174; 20 TCID₅₀ was equivalent to one 50% macaque infectious dose (MID₅₀).

Monkeys and virus inoculation. Ten adult (5- to 8-year-old) rhesus macaques (*Macaca mulatta*), which were of Chinese origin, were used in this study. All monkeys used were treated in accordance with the institutional regulations approved by the Committee for Experimental Use of Non-human Primates in the Institute for Virus Research, Kyoto University. Eight monkeys were anaesthetized by intramuscular injection of ketamine chloride and inoculated intrarectally with 2 × 10³ TCID₅₀ SHIV-C2/1-KS661c. All intrarectal inoculations were done with a paediatric feeding catheter 10 cm from the anus. The catheter was inserted carefully to avoid causing

trauma. Two monkeys were euthanized at each of 3 (animals MM301 and MM307), 6 (MM300 and MM309), 13 (MM313 and MM334) and 27 (MM308 and MM310) days *p.i.* Two monkeys (MM244 and MM314) were used as uninfected controls.

Sample collection. Blood was collected periodically from all monkeys. Peripheral blood mononuclear cells (PBMCs) and plasma were separated from heparinized blood by Percoll (Lymphocyte Separation Solution; Nacalai Tesque) density-gradient centrifugation. Plasma was frozen at −80 °C until use. Complete sets of organs were obtained at the time of euthanasia. Parts of the samples were frozen directly at −80 °C until further use (i.e. quantification of proviral DNA). Residual samples of spleen, thymus, and axillary, inguinal and mesenteric lymph nodes were minced and filtered through a 40 µm nylon filter (Becton Dickinson). Samples of jejunum and rectum were washed in Dulbecco's modified Eagle's medium (DMEM) containing 0.45 mM dithiothreitol, cut into 1 cm² pieces and agitated in DMEM medium containing 5% fetal calf serum (FCS) for 1 h at room temperature. After short sedimentation, supernatants and tissue fragments were processed to give intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL), respectively. The supernatants (containing iEL) were filtered through columns containing packed glass wool and centrifuged at 1600 r.p.m. for 7 min; pellets were then suspended in 30% Percoll (Pharmacia) and centrifuged at 1800 r.p.m. for 20 min. The resulting pellets were resuspended in 44% Percoll, layered on 70% Percoll and centrifuged at 1800 r.p.m. for 20 min. Cells at the interface between the 44 and 70% Percoll layers were collected. The residual tissue fragments were agitated in Hanks' buffer containing 5 mM EDTA for 10 min at room temperature and the supernatants were removed. This step was repeated three times. The fragments were suspended in RPMI 1640 medium (Gibco) containing 10% FCS and, after agitation for 30 min at room temperature, the supernatants were removed. The fragments were resuspended in RPMI 1640 medium containing 10% FCS and type II collagenase (0.2 mg ml^{−1}; Sigma) and agitated for 90 min at room temperature. The suspensions (containing LPL) were filtered through glass-wool columns and cells were enriched by Percoll density-gradient centrifugation as described above for iEL. The cells obtained from each organ were used immediately in the infectious plaque assay and flow-cytometry analysis.

Quantification of plasma viral RNA. The viral RNA loads in plasma were determined by quantitative RT-PCR (Suryanarayana *et al.*, 1998). Total RNAs were prepared from plasma with a QIAamp Viral RNA kit (QIAGEN). RT-PCR was performed with a Taqman EZ RT-PCR kit (Perkin Elmer) for the SIV *gag* region using the following primers: SIV2-696F (5'-GGAAATTACCCAGTACAACAA-ATAGG-3') and SIV2-784R (5'-TCTATCAATTTTACCCAGGCAT-TTA-3'). A labelled probe, SIV2-731T (5'-Fam-TGTCCACCTGCC-ATTAAGCCCG-Tamra-3'; Perkin Elmer), was used for detection of the PCR products. These reactions were performed with a Prism 7700 Sequence Detector (Applied Biosystems) and analysed by using the manufacturer's software. For each run, a standard curve was generated from dilutions whose copy numbers were known and the RNA in the plasma samples was quantified based on the standard curve.

Quantification of proviral DNA. Proviral DNA loads in tissues were determined by quantitative PCR. DNA samples were extracted directly from frozen tissues with a Qiagen DNeasy Tissue kit. PCR was performed with a Taqman PCR Reagent kit (Perkin Elmer) using the same primer set and probe used in RT-PCR. A standard curve was generated from a plasmid DNA sample containing the full genome of SHIV-NM-3rN, which was quantified with a UV spectrophotometer.

Infectious plaque assay. Infectious virus was quantified and isolated by using an infectious plaque assay (Kato *et al.*, 1998). An

agarose-gel bilayer containing RPMI 1640 medium was made in plastic culture dishes with a diameter of 100 mm; the lower layer consisted of 12 ml 1.2% agarose (Agarose NA; Pharmacia) and the upper layer consisted of 12 ml 0.4% low gelling-temperature agarose (SeaPlaque Agarose; FMC). Dishes were incubated at 37 °C in 5% CO₂ overnight. The following day, 2 × 10⁶ cells of each sample and 8 × 10⁶ M8166 cells (Clapham *et al.*, 1987) were suspended in 3 ml 0.4% low gelling-temperature agarose solution containing the culture medium and the mixture was immediately overlaid on the agarose-gel layer prepared previously. After the gel had hardened, plates were covered with 12 ml culture medium and incubated at 37 °C in 5% CO₂ for 10 days. The medium over the plates was replaced with fresh medium every day. After removal of the medium on day 10, plates were stained with 2 ml 0.7% MTT for 2 h to count the number of plaques.

Flow-cytometry analysis. The frequencies of CD4⁺ single-positive and CD4CD8 double-positive T cells in PBMCs and various tissues were examined by flow cytometry. Lymphocytes were treated with anti-CD3 (FN-18–fluorescein isothiocyanate; Biosource), anti-CD4 (Nu-TH/1–phycoerythrin; NICHIREI) and anti-CD8 (SK1–PerCP; Becton-Dickinson) monoclonal antibodies and examined on a FACScan analyser (Becton Dickinson). The absolute number of lymphocytes in the blood was determined by using an automated blood-cell counter (F-820; Sysmex).

RESULTS

Intrarectal infection of macaque monkeys with SHIV-C2/1-KS661c

Eight rhesus macaque monkeys were inoculated intrarectally with SHIV-C2/1-KS661c and two monkeys were euthanized at each of 3, 6, 13 and 27 days p.i. In the two monkeys that were euthanized at 3 days p.i. (MM301 and MM307), plasma viral RNA was not detected in the time between inoculation and euthanasia. However, plasma viral RNA was first detected at 3 days p.i. in one monkey (MM300) and at 6 or 7 days p.i. in five monkeys (MM309, MM313, MM334, MM308 and MM310) (Fig. 1). The plasma viral RNA load of these monkeys reached peak levels, about 10⁸ to 5 × 10⁹ copies ml⁻¹, at 13 days p.i. and then decreased, reaching 10⁶ copies ml⁻¹ at 27 days p.i. CD4⁺ T-cell counts in peripheral blood of these monkeys started to decrease from day 6 p.i. and were lower than 500 cells μl⁻¹ by 13 days p.i. (Fig. 2). These low counts of CD4⁺ T cells remained at the same levels until 27 days p.i.

Detection of proviral DNA in various tissues early after intrarectal inoculation

To investigate virus distribution to the systemic tissues early after infection, proviral DNAs in various tissues were determined by quantitative PCR. Proviral DNA was already detected at 3 days p.i. in the rectum and distal lymphoid tissues (thymus and axillary lymph node) of one monkey examined at this time (MM301) (Fig. 3). It was also detected in non-lymphoid tissues (kidney and lung; data not shown) at low levels [< 20 copies (μg DNA)⁻¹]. These results show that the virus spread quickly to the systemic tissues after intrarectal inoculation. In both monkeys that were examined at 6 days p.i. (MM300 and MM309), proviral

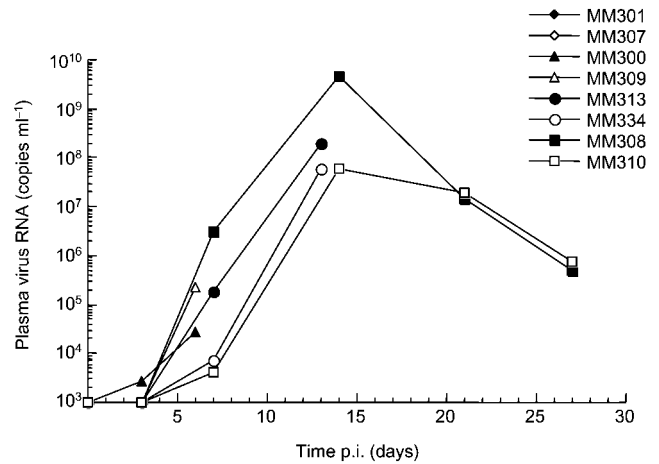


Fig. 1. Plasma viral RNA loads of eight monkeys inoculated intrarectally with SHIV-C2/1-KS661c. MM301 and MM307, MM300 and MM309, MM313 and MM334, and MM308 and MM310 were euthanized at 3, 6, 13 and 27 days p.i., respectively. The detection limit of this assay was 1 × 10³ copies ml⁻¹.

DNA was detected in PBMCs as well as other lymphoid tissues (Fig. 3), suggesting that detectable levels of infected cells had drained to the peripheral bloodstream by this time. However, the titres of proviral DNA in these tissues were less than about 10² copies (μg DNA)⁻¹ and proviral DNA was not detected in some tissues at 6 days p.i. In monkeys examined at 13 days p.i. (MM313 and MM334), when viraemia reached peak levels, proviral DNA was detected in all tissues examined and virus titres in each tissue were much higher than those determined at 6 days p.i. In most of the lymphoid tissues and the intestinal tract, proviral DNA was detected at > 10³ copies μg⁻¹ (Fig. 3). In the non-lymphoid tissues of these monkeys, including lung, liver, kidney and

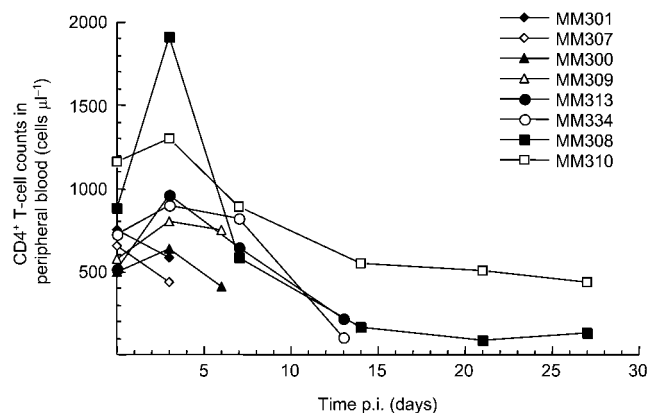


Fig. 2. Number of CD4⁺ T cells in peripheral blood of eight monkeys inoculated intrarectally with SHIV-C2/1-KS661c. MM301 and MM307, MM300 and MM309, MM313 and MM334, and MM308 and MM310 were euthanized at 3, 6, 13 and 27 days after inoculation, respectively.

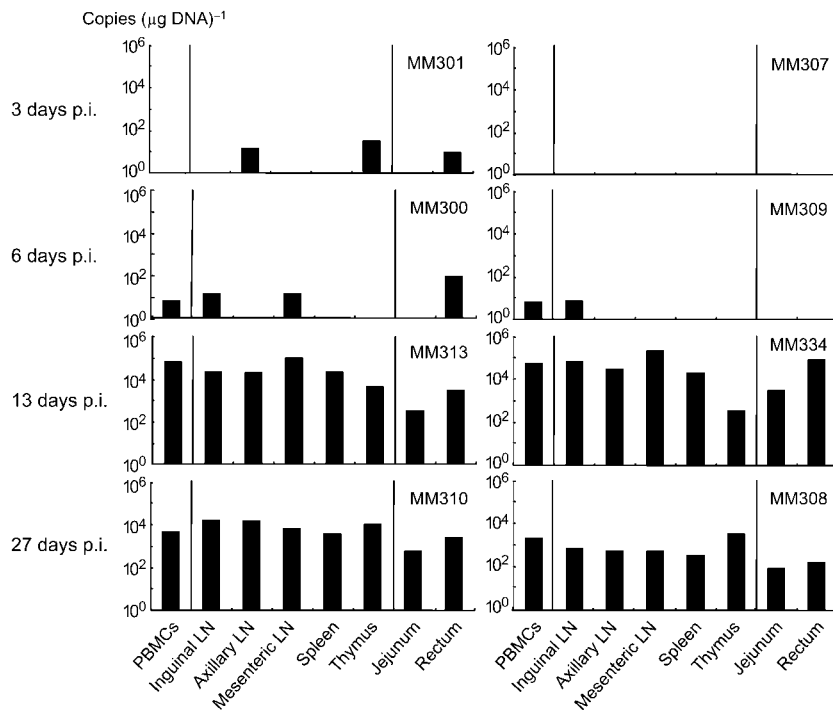


Fig. 3. Proviral DNA loads in various tissues of eight SHIV-C2/1-KS661c-inoculated monkeys. Virus loads were determined by quantitative PCR and are expressed as viral DNA copy numbers (μg total DNA extracted from tissue homogenates) $^{-1}$.

brain, proviral DNA was detected at about $10\text{--}10^3$ copies μg^{-1} (data not shown). The titres of proviral DNA in all tissues of the monkeys examined at 27 days p.i. (MM308 and MM310) were still $>10^3$ copies μg^{-1} (MM310) or 10^2 copies μg^{-1} (MM308) (Fig. 3). These results show that virus infections in various tissues were amplified from 6 to 13 days p.i. and then decreased, but virus remained detectable in each tissue until 27 days p.i.

Detection of infectious virus in various tissues early after intrarectal infection

Although proviral DNA was present in each tissue early after infection, it was not clear whether these tissues released infectious virus. To observe the release of infectious virus from various tissues, infectious virus only was quantified in these tissues by using an infectious plaque assay. Infectious virus was first detected at 6 days p.i. in inguinal and mesenteric lymph nodes of two monkeys (MM300 and MM309) at 1.0 and 0.5 p.f.u. per 10^6 cells, respectively (Fig. 4). Thereafter, the levels of infectious virus increased dramatically and high titres of infectious virus were detected in many lymphoid tissues of monkeys examined at 13 days p.i. (MM313 and MM334) (Fig. 4). In both of these monkeys, the highest numbers of infectious virus (119 and 100 p.f.u. per 10^6 cells, respectively) were detected in the mesenteric lymph nodes, suggesting that this is the main site of production of infectious virus. Levels of infectious virus in the axillary and inguinal lymph nodes of these monkeys were 55.5–100.0 p.f.u. per 10^6 cells. In MM313, levels of infectious virus in PBMCs and thymus (99.5 and 110.0 p.f.u. per 10^6 cells, respectively) were almost as high as those in the mesenteric lymph node.

However, MM334 tissues had remarkably low numbers of infectious virus in PBMCs and thymus (24.0 and 3.5 p.f.u. per 10^6 cells, respectively). In the monkeys examined at 27 days p.i. (MM308 and MM310), infectious virus was detected at very low levels in lymphoid tissues (<19 p.f.u. per 10^6 cells) (Fig. 4), whereas proviral DNA was detected at the same levels as at 13 days p.i. (Fig. 3). These results suggest that, after the peak of viraemia, high levels of virus existed in the lymphoid tissues, but most virus did not replicate there. In particular, PBMCs and thymus contained infectious virus at only 0–5 p.f.u. per 10^6 cells at 27 days p.i., suggesting that these tissues hardly contribute to the release of infectious virus after the peak of viraemia.

In the intestinal tract, infectious virus was hardly detected throughout the infection (Fig. 4). At 13 days p.i., some infectious virus was detected in the jejunum, but titres were much lower than those in the lymphoid tissues (4.5 and 12.5 p.f.u. per 10^6 cells in the jejunum of MM313 and MM334, respectively). These results show that virus replication was much lower in the intestinal tract than in the lymphoid tissues at the early phase of infection, although the virus reached the intestinal tract at the same time that it reached the lymphoid tissues.

Sequential changes in the proportion of CD4⁺ T cells in various tissues early after intrarectal infection

CD4⁺ T cells have been reported as the main target and source for amplification of the virus. To estimate the effect of virus replication on the proportion of CD4⁺ T cells existing in various tissues, sequential changes in the

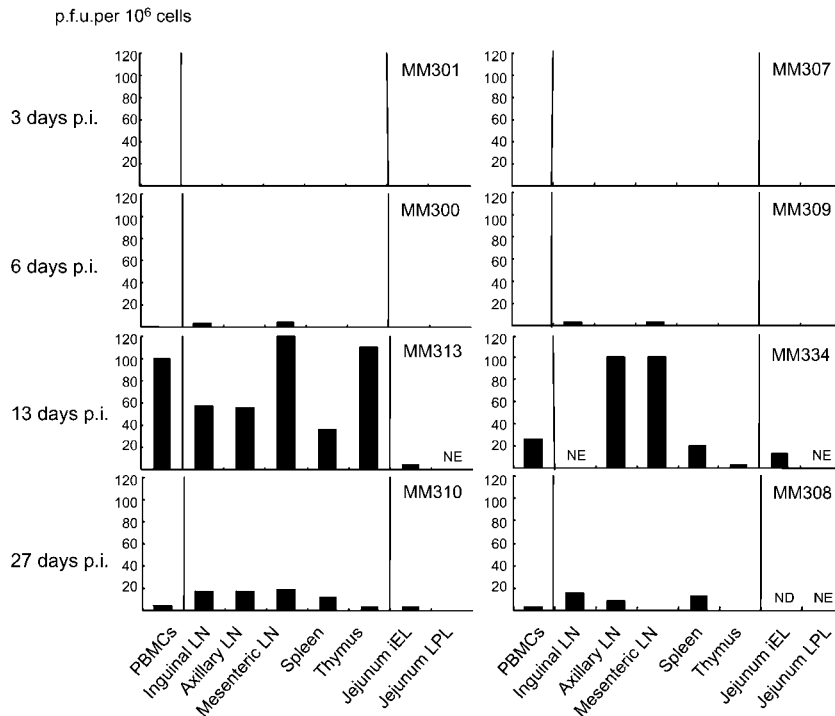


Fig. 4. Infectious virus loads in various tissues of eight SHIV-C2/1-KS661c-inoculated monkeys. Virus loads were determined by infectious plaque assay and are expressed as p.f.u. per 10^6 cells. ND, Assay was not done because not enough lymphocytes were obtained; NE, culture was not evaluated because of contamination.

proportion of $CD4^+$ T cells were examined in each tissue in which virus was detected at various loads by using flow cytometry. The mean percentages of $CD4^+$ T cells in PBMCs, spleen, thymus, and inguinal, axillary and mesenteric lymph nodes of uninfected controls (MM244 and MM314) were 35, 26, 43, 59, 56 and 58 % of total lymphocytes, respectively (Fig. 5). The percentages of $CD4^+$ T cells in PBMCs were higher in monkeys at 6 and 13 days p.i. (62 and 61 % of total lymphocytes, respectively) than in the uninfected normal controls, but then decreased to 12 % of total lymphocytes by 27 days p.i. In other lymphoid tissues, the percentages of $CD4^+$ T cells remained at the level of uninfected normal controls until 6 days p.i. Between 6 and 27 days p.i., the percentages of $CD4^+$ T cells decreased significantly to 9–14 % of whole lymphocytes in each tissue.

In uninfected controls (MM244 and MM314), the percentages of $CD4^+$ T cells in the intestinal tract were lower than those in the lymphoid tissues. iEL and LPL were examined separately, because it was previously reported that the proportions of the major intestinal T-cell subsets differed markedly between the iEL and LPL (Veazey *et al.*, 1997, 2000a, b) and it was expected in the present study that the infection kinetics in iEL and LPL would differ. The mean percentages of $CD4^+$ T cells in the jejunum of control monkeys were 10 % in iEL and 34 % in LPL, and those in the rectums were 5 % in iEL and 11 % in LPL (Fig. 5). $CD4^+$ T cells in the intestinal tract remained at the same level as those in the uninfected controls until 13 days p.i. and then decreased to 1–2 % of whole lymphocytes by 27 days p.i. These sequential changes of $CD4^+$ T cells were almost the same in iEL and LPL of the jejunum and rectum. Thus,

SHIV-C2/1 caused marked $CD4^+$ T-cell depletion both in peripheral blood and the intestinal tract. The extent of $CD4^+$ T-cell depletion in intestinal tract and lymphoid tissues correlated with the extent of virus replication in each tissue.

Sequential changes in the proportion of $CD4$ single-positive (SP) and $CD4CD8$ double-positive (DP) T cells in the jejunum and thymus

There were larger percentages of $CD4CD8$ DP T cells in the jejunum than in the lymphoid tissues, apart from the thymus. In the jejunum of the normal control monkeys, the mean percentages of $CD4CD8$ DP T cells in total $CD4^+$ T cells were 64 % in iEL and 45 % in LPL, whereas in the lymphoid tissues, they were only 8–16 % (data not shown). The proportion of $CD4$ SP T cells in the jejunum remained at the level of uninfected controls until 13 days p.i. (15 and 34 % in jejunum iEL and LPL, respectively, at 13 days) and then dropped sharply to <0.3 % in both iEL and LPL by 27 days p.i. This sequential change in the proportion of $CD4$ SP T cells in the jejunum was the same as that observed for total $CD4^+$ T cells. However, the proportion of $CD4CD8$ DP T cells started to decrease from day 3 p.i.; at 13 days p.i., it was <5 % in both iEL and LPL of the jejunum (Fig. 6).

In the thymus of the uninfected control monkeys, 91 % of $CD4^+$ T cells were $CD4CD8$ DP T cells (40 % of total lymphocytes). Moreover, the thymus had many $CD3^-$ $CD4CD8$ DP cells (45 % of total lymphocytes). In the thymus, $CD3^+$ $CD4CD8$ DP cells tended to become depleted first, followed by $CD3^+$ $CD4$ SP cells and then $CD3^-$ $CD4CD8$ DP cells (Fig. 7). These results

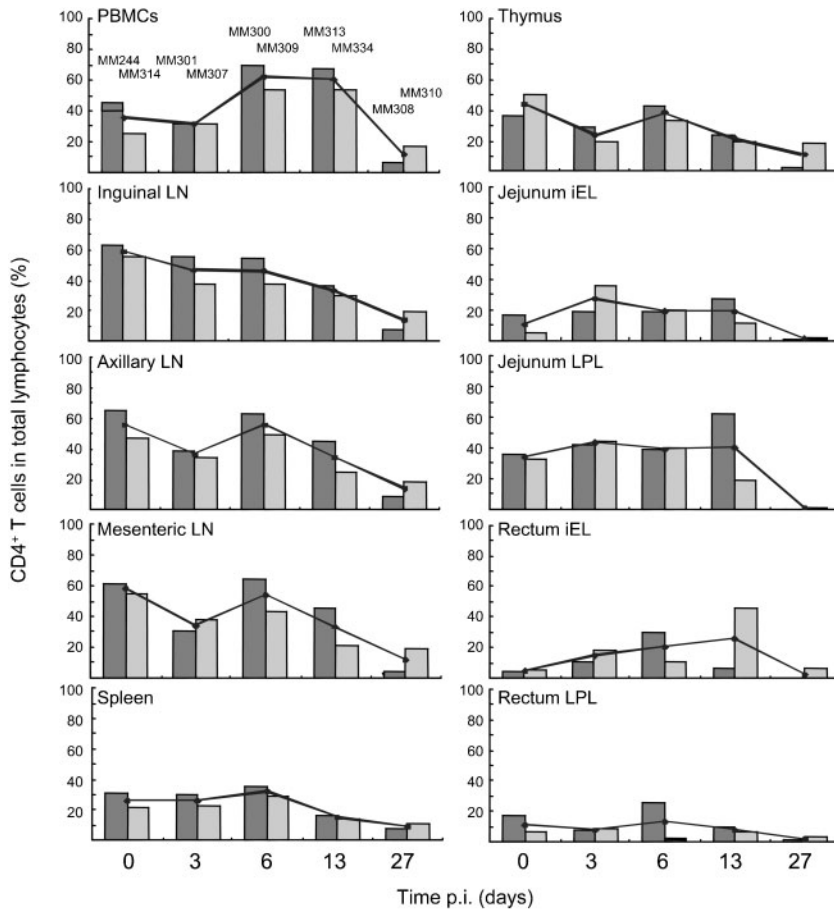


Fig. 5. Sequential changes in the proportion of CD4⁺ T cells in the various tissues early after intrarectal infection. The percentage of CD4⁺ T cells in total lymphocytes was determined by flow cytometry. Each bar represents one monkey. The lines indicate the mean values of two monkeys at each time point.

suggest that there is a difference in the effect of virus infection on CD4 SP T cells, CD4CD8 DP T cells and CD3⁻CD4CD8 DP cells early after intrarectal infection.

DISCUSSION

In this study, to observe the early virological events in various tissues after mucosal infection, SHIV-C2/1-KS661c was used to inoculate rhesus monkeys intrarectally and

proviral DNA and infectious viruses were quantified in various tissues by quantitative PCR and infectious plaque assay, respectively. At 3 days p.i., proviral DNA was already present in not only the rectum, but also the thymus, axillary lymph node, kidney and lung. These results suggested that the intrarectally inoculated virus spread quickly to the systemic tissues. Hu *et al.* (2000) showed that SIV penetrated the vaginal mucosa of rhesus macaques within 60 min of intravaginal inoculation, infecting primarily intraepithelial

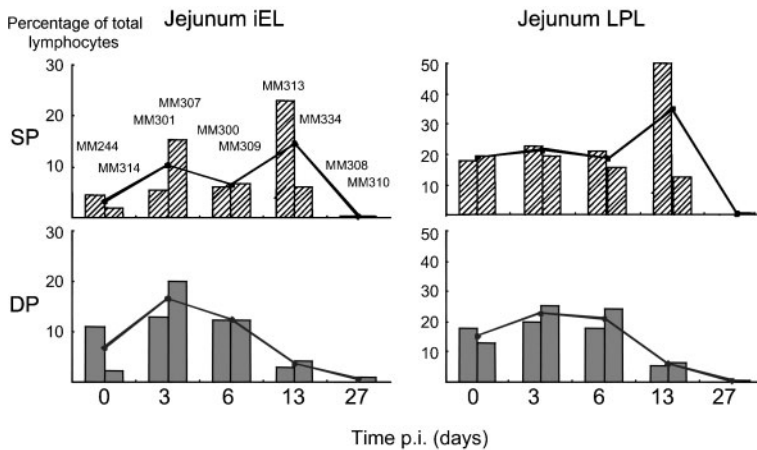


Fig. 6. Sequential changes in the proportion of CD4 single-positive (SP) and CD4CD8 double-positive (DP) T cells in the jejunum iEL and LPL. The percentage of CD4⁺ T cells in total lymphocytes was determined by flow cytometry. Each bar represents one monkey. The lines indicate the mean values of two monkeys at each time point.

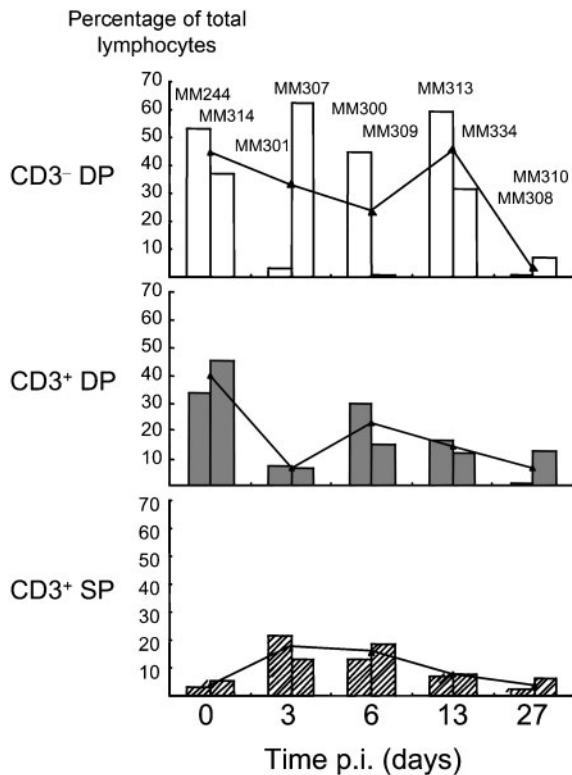


Fig. 7. Sequential changes in the proportion of CD4 single-positive (SP) and CD4CD8 double-positive (DP) T cells in the thymus. The percentage of CD4⁺ T cells in total lymphocytes was determined by flow cytometry. Each bar represents one monkey. The lines indicate the mean values of two monkeys at each time point.

dendritic cells (DCs), and that SIV-infected cells were detected in iliac lymph nodes within 18 h of inoculation. Because the epithelium of the rectum mucosa is just as rich in DCs as the vaginal mucosa, it appears that the virus is transported to the draining lymph nodes by DCs within several hours of intrarectal exposure in this study. Once the virus reaches the local lymphoid tissues, systemic dissemination might occur shortly thereafter.

Virus levels increased remarkably between 6 and 13 days p.i. and high levels of proviral DNA and infectious virus were detected in various lymphoid tissues at 13 days p.i., which was the time of peak viraemia. Among all the tissues examined, the mesenteric lymph node had the largest level of infectious virus. This result is consistent with a previous study that showed that mesenteric lymph nodes contain numerous SIV-infected cells in the early stages of SIV infection (Cantó-Nogués *et al.*, 2001; O'Neil *et al.*, 1999). The intestinal tract is constantly exposed to antigens in foods and pathogens. Therefore, the mesenteric lymph node, which is a draining lymph node of the intestinal tract, might have many more activated T cells than other lymphoid tissues. Because SIV/HIV-1 can replicate optimally in

activated T cells, the mesenteric lymph nodes might release the largest numbers of infectious virus.

After the peak of viraemia, the titre of infectious virus in the lymphoid tissues decreased significantly. Around this time, it is generally recognized that adaptive immunity is induced in the host. Therefore, the induction of such acquired immunity might also result in the suppression of virus replication in the lymphoid tissues. Moreover, CD4⁺ T cells, which are the main target and source of amplification of the virus (Dalglish *et al.*, 1984; Klatzmann *et al.*, 1984; Sattentau *et al.*, 1988), were depleted in the lymphoid tissues by this time, thus resulting in the low level of virus production there. In contrast, significant proviral DNA remained in the lymphoid tissues after the peak of viraemia. The identity of the cells holding this proviral DNA was not clear, but they might represent a reservoir pool of virus until the development of AIDS.

In the intestinal tract, infectious virus was detected, but the virus load was much lower than in the lymphoid tissues. This is surprising because it was expected that the intestinal tract would have as many activated lymphocytes as the mesenteric lymph node and the virus replicates efficiently in those cells. Some reasons for the low titre of infectious virus in the intestinal tract were considered. Firstly, the sample of intestinal tract, which was separated as iEL and LPL, contains various types of cells and the percentage of CD4⁺ T cells there was much lower than in samples of lymphoid tissues, thus giving rise to a lower level of virus production in the intestinal tract. In addition, there is a possibility that the intestinal tract has a strong immunity. Following virus infection, the components of innate immunity might respond rapidly and provide time for the subsequent development of adaptive immunity. Natural killer (NK) cells, which are a critical component of innate immunity to virus infection, were reported to mediate suppression of HIV-1 replication by producing CC chemokines or causing cytotoxicity against HIV-1-infected cells (Baum *et al.*, 1996; Fehniger *et al.*, 1998; Kottitil *et al.*, 2003; Levy, 2001; Oliva *et al.*, 1998). In the monkeys used in this study, NK activity using K562 target cells was measured in PBMCs, intestinal tract and inguinal and mesenteric lymph nodes (K. Ibuki, N. Saito, Y. Enose, A. Miyake, H. Suzuki, R. Horiuchi, T. Miura & M. Hayami, unpublished data). Among these tissues, NK activity was much higher in the intestinal tract of both normal and infected monkeys. This result raises the possibility that NK activity in the intestinal tract contributed to the suppression of virus replication in the present study.

In the lymphoid tissues, levels of CD4⁺ T cells decreased significantly from day 6 p.i. In contrast, in the intestinal tract, CD4⁺ T cells remained at the same level as in uninfected normal controls until 13 days p.i. These results clearly correlated with the extent of virus replication in the lymphoid tissues and intestinal tract. However, CD4⁺ T cells in the intestinal tract were finally depleted by 27 days p.i. In previous studies, it was reported that the target tissues or organs of the virus differed when using CXCR4 or CCR5 as

co-receptors of virus entry (Harouse *et al.*, 1999; Reyes *et al.*, 2004). In the early phase of infection, CXCR4-utilizing SHIV causes rapid depletion of CD4⁺ T cells in the peripheral blood, but not in the intestinal tissues, whereas CCR5-utilizing SHIV causes rapid depletion of CD4⁺ T cells in the intestinal tissues, but not in the peripheral blood. Recent studies using SIV-infected monkeys showed a profound and selective loss of memory CD4⁺ CCR5⁺ T cells in the intestinal tract in the early phase of infection (Brenchley *et al.*, 2004; Mehandru *et al.*, 2004; Mattapallil *et al.*, 2005; Li *et al.*, 2005). Moreover, some HIV-1-carrier studies demonstrated that a significant and preferential depletion of mucosal CD4⁺ T cells that express CCR5 occurs compared with peripheral blood or lymphoid tissues (Veazey *et al.*, 2000a, b; Centlivre *et al.*, 2005). SIV and most primary isolated HIV-1 utilize CCR5 as co-receptor for entry, and target tissues of dual-tropic virus using both CXCR4 and CCR5 were unknown. In this study, it was shown that SHIV-C2/1 using both CXCR4 and CCR5 as co-receptors caused rapid CD4⁺ T-cell depletion in both peripheral blood and the intestinal tract.

Among the tissues examined, the thymus and intestinal tract had a large percentage of CD4CD8 DP T cells. Both tissues have been reported as sites of maturation of lymphocytes (Haynes *et al.*, 1990; Lundqvist *et al.*, 1995) and CD4CD8 DP T cells have been proposed to be immature T cells. A previous study reported that CD4CD8 DP T cells in the thymus were susceptible to HIV-1 infection (Schnittman *et al.*, 1990). Moreover, CD4 SP and CD4CD8 DP T cells were found to decrease at the same time during acute SIV infection in rhesus macaques in the thymus (Rosenzweig *et al.*, 2000) and intestinal tract (Mattapallil *et al.*, 2000; Smit-McBride *et al.*, 1998). In this study, however, CD4CD8 DP T cells started to decrease earlier than mature CD4 SP T cells in both the thymus and intestinal tract, suggesting that the resultant effect of virus infection is different between the mature and immature T cells in each tissue. CD4CD8 DP T cells were observed to decrease in both tissues before virus replication. This shows that the virus may indirectly kill CD4CD8 DP T cells. Moreover, CD3⁻ CD4CD8 DP cells, which are precursors of CD3⁺ CD4CD8 DP cells (Hori *et al.*, 1991), were also depleted in the thymus after the peak of viraemia. Further studies of the pathogenesis of virus infection in immature T cells of the thymus and intestinal tract may lead to better understanding of the mechanisms of CD4⁺ cell depletion in HIV-1-infected humans.

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